USE OF A POLYPEPTIDE FOR DETECTING, PREVENTING OR TREATING A PATHOLOGICAL CONDITION ASSOCIATED WITH A DEGENERATIVE, NEUROLOGICAL OR AUTOIMMUNE DISEASE

BACKGROUND

The present invention relates in particular to the use of at least one polypeptide to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for for detecting, preventing or treating a pathological condition associated with a degenerative and/or autoimmune and/or neurological disease.

According to the invention, the expression degenerative disease is understood to mean a disease in which a process of cell death or of cell destruction is associated with physiological and/or clinical disorders. Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease are classified amongst neurogenerative diseases. The expression autoimmune disease is understood to mean a hyperactivity of the immune system toward one or more autoantigens. Multiple sclerosis (MS), rheumatoid arthritis (RA) and lupus erythematosus are classified among autoimmune diseases.

Multiple sclerosis is a chronic disease of the central nervous system in humans which progresses through a succession of phases of remission and of flare-up or in a regular progression and whose anatomicopathological characteristic consists in the formation of well delimited demyelination zones in the white substance of the brain and of the spinal cord.

At the histological level, these zones exhibit, at the early stage of the lesional process, a degradation of the periaxonal myelin associated with an impairment of the glial cells responsible for this demyelination.

Inflammatory macrophage activation causing the microglial cells (resident tissue macrophages of the central nervous system), as well as, probably, macrophages from infiltrated blood monocytes, is associated with this demyelination process and contributes to the destruction of the myelinated sheets. At the center of the demyelinated zone, there is a relative depletion of glial cells whereas a proliferation of astrocytes develops at the periphery and can invade the demyelinated plaque in order to generate a fibrous or gliotic plaque. These sclerotic structures are responsible for the name given to the disease.

Another characteristic of these plaques is their almost systematic association with a vascular element around which they develop.

At the histological level, a frequent alteration of the blood-brain barrier (BBB) consisting of capillary endothelium is observed. One of the key elements in maintaining the BBB consists of the underlying presence of cytoplasmic extensions of the astrocytes, called astrocytic feet. Possibly, the astrocytic feet induce the formation or allow the maintenance of tight joining structures which ensure the cohesion of the capillary endothelial barrier concretizing the BBB. However, various pathological models report the alteration of the BBB and a depletion of the astrocytic feet.

Moreover, in the lesional process in MS, the alteration of the BBB contributes toward amplifying the associated inflammatory response by the influx of lymphoid cells from the bloodstream. The contribution of the inflammation associated with the immune cells is important in MS and participates in the lesional process.

The etiology of MS is the source of a current debate because the disease could have various origins. Hypotheses have been emitted on a bacterial and/or viral origin. Moreover, as described in patent application WO 95/21859, H. Perron et al. have been led to investigate one or more effector agents for the pathogenic process resulting in the typical formation of demyelination plaques and in astrocytic gliosis. In the context of this study, they demonstrated the presence, in the cerebrospinal fluid (CSF) and the serum of MS patients, of at least one factor which exhibits a toxic activity toward human or animal astrocyte and oligodendrocyte cells. This toxic activity is characterized by a cytomorphological disorganization of the network of intermediate filaments and/or a degradation of the proteins of said filaments and/or a cell death by apoptosis of the glial cells. They established a significant correlation between the *in vitro* detection of this toxic activity in samples of CSF and of serum of MS patients and multiple sclerosis by a quantitative colorimetric assay with methyltetrazolium bromide (MTT) of the live cells, as described in patent application WO 95/21859. Moreover, C. Malcus-Vocanson et al. have shown that urine is a very favorable biological fluid for the detection of the activity of this toxic factor and developed a method using flow cytometry to detect and/or quantify the adherent glial cells which are dead through apoptosis. All the information relating to this method is described in patent application WO 98/11439, whose content is incorporated by way of reference.

Trials were carried out starting with a protein fraction of CSF and of urine from MS patients in order to try to identify this toxic factor. The protein content of each fraction was separated on a 12% SDS-PAGE gel and observed after silver staining of the gel. Among the proteins observed, a protein fraction centered over an apparent molecular weight of about

21 kD was found not predominantly associated with the toxic activity detected *in vitro* and a fraction centered over an apparent molecular weight of about 17 kD was found predominantly associated with the toxic activity.

Injection of the fraction from the SCF of MS patients into the brain of Lewis rats and postmortem histological observation of brain sections of the rats made it possible to observe, three months after the injection, an apoptosis of the astrocytic population and the formation of demyelination plaques. All the information is contained in patent application WO 97/33466, whose content is incorporated by way of reference. These observations are in accordance with those which have been made on the brain sections of patients suffering from MS, after biopsy (N. Benjelloun et al. Cell. Mol. Biol., 1998, 44(4), 579-583).

SUMMARY

The present inventors have now identified and analyzed the proteins associated with this toxic activity toward glial cells in biological samples from MS patients, in particular in urine, cerebrospinal fluid and serum.

After purification of the proteins and separation on SDS-TRICINE gel, the inventors have demonstrated the presence of four bands of interest having different apparent molecular weights, of 8, 14, 18 and 20 kD respectively, corresponding to at least five different protein families. The proteins of these families were then analyzed by mass spectrometry and/or sequencing and a search for homology in data banks (NCBI, Basic Blast Search, Protein Blastp, the protein sequences are entered in a FASTA format into the nr database, the algorithm used is Matrix BLOSUM62, the identity called "Identities" corresponds to the number of identical amino acids, given as a percentage, and the positivity

"Positives" corresponds to the amino acids exhibiting biological equivalence according to the abovementioned parameters of the software, given as a percentage). These proteins belong to the protein families of Perlecan, of the precursor of the retinol-binding plasma protein, of the GM2 activator protein, of calgranulin and of saposin B. More precisely, the proteins are (i) for the 20 kD band, the C-terminal fragment of Perlecan which starts at amino acid 3464 and ends at amino acid 3707 (Murdoch AD et al. J Biol Chem, 1992, April 25; 267 (12):8544-47), and designated by a reference in the sequence identifier SEQ ID No. 2 (the full-length Perlecan protein being designated by a reference in SEQ ID No. 1), (ii) for the 20 kD band, the precursor of the retinol-binding plasma protein (Monaco HL et al., Science, 1995, 268 (5213):1039-1041) whose sequence is given in SEQ ID No. 4, (iii) for the 18 kD band, the GM2 activator protein (Furst W et al., Euro J Biochem, 1990, Sep 24; 193(3):709-14) identified in SEQ ID No. 8, (iv) for the 14 kD band, calgranulin B (Lagasse. E et al., Mol Cell Biol, 1988, Jun; 8(6):2402-10) identified in SEQ ID No. 17 and (v) for the 8 kD band, saposin B (Kleinschmidt T et al., Biol Chem Hoppe Seyler, 1988, Dec; 369(12):1361-5) represented in SEQ ID No. 24. They have also demonstrated the presence of variant sequences to said reference sequences, in particular for the 18 kD band a variant sequence of the GM2 activator protein designated by the reference SEQ ID No. 9. These variant protein sequences are the product of mutations at the level of the genes encoding said proteins or are the result of splicing phenomena. It should be noted, for example, that calprotectin is a variant of calgranulin B.

The C-terminal fragment of the Perlecan protein (SEQ ID No. 2) is encoded, for example, by the DNA nucleotide sequence SEQ ID No. 69, taking into account the genetic code. The precursor protein for the retinol-binding

plasma protein (SEQ ID No. 4) is encoded, for example, by the DNA nucleotide sequence SEQ ID No. 70, taking into account the genetic code. The GM2 activator protein (SEQ ID No. 8) is encoded, for example, by the DNA nucleotide sequence SEQ ID No. 31, taking into account the genetic code. The peptides FSWDNCFEGK DPAVIR and YSLPKSEFAV PDLELP derived from the GM2 activator mutated polypeptide (SEQ ID No. 9) are encoded by the DNA nucleotide sequences SEQ ID No. 66 and SEQ ID No. 67, respectively, taking into account the genetic code. The calgranulin B protein (SEQ ID No. 17) is encoded, for example, by the DNA nucleotide sequence SEQ ID No. 42, taking into account the genetic code. The saposin B protein (SEQ ID No. 24) is encoded, for example, by the DNA nucleotide sequence SEQ ID No. 53, taking into account the genetic code.

The expression protein family is understood to mean all the proteins encoded from the same DNA gene and which result from a differential multiple splicing of the gene and/or of a different reading frame. The DNA gene is transcribed with alternative splicing phenomena, leading to the translation of different primary sequences of proteins. All these proteins belong to the same protein family. The term "protein family" also includes proteins which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with a reference protein sequence of the family.

The expression multiple splicing is understood to mean a splicing occurring at least once in the nucleotide region of interest.

For example, the expression precursor protein family for the retinol-binding plasma protein designates the protein family comprising *at least* the proteins or fragments of proteins having the sequence SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, and the

proteins encoded by the corresponding gene according to different reading frames.

For example, the expression GM2 activator protein family designates the protein family comprising *at least* the proteins or fragments of proteins having the sequence SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14,, SEQ ID No. 15, SEQ ID No. 16, and the proteins encoded by the corresponding gene according to different reading frames, which result from a differential multiple splicing of the gene and/or of a different reading frame.

For example, the expression calgranulin B protein family designates the protein family comprising *at least* the proteins or fragments of proteins having the sequence SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, and the proteins encoded by the corresponding gene according to different reading frames, which result from a differential multiple splicing of the gene and/or of a different reading frame. The proteins MRP14 (SEQ ID No. 17) and MRP8 (SEQ ID No. 18) have a different protein sequence while being encoded by the same gene; they belong to the same protein family.

For example, the expression saposin B protein family designates the protein family comprising *at least* the proteins or fragments of proteins having the sequence SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, and the proteins encoded by the corresponding gene according to different reading frames, which result from a differential multiple splicing of the gene and/or of a different reading frame.

The expression nucleic acid family encoding a protein is understood to mean all the cDNA and/or RNA nucleic

sequences transcribed from the same DNA gene and which result from a differential multiple splicing. The DNA gene is transcribed with differential splicing phenomena and leads to the synthesis of different nucleic acids (cDNA, RNA) of different sequences. All these cDNA and mRNA sequences are considered to belong to the same nucleic acid family.

For example, the expression nucleic acid family encoding the precursor protein family for the retinol-binding plasma protein designates the nucleic acid family comprising *at least* the nucleic acids or fragments having the sequence SEQ ID No. 30.

For example, the expression nucleic acid family encoding the GM2 activator protein family designates the nucleic acid family comprising *at least* the nucleic acids or fragments having the sequences SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 34, SEQ ID No. 35, SEQ ID No. 36, SEQ ID No. 37, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 40, SEQ ID No. 41 which result from a differential multiple splicing of the gene and/or of a different reading frame.

For example, the expression nucleic acid family encoding the calgranulin B protein family designates the nucleic acid family comprising *at least* the nucleic acids or fragments having the sequences SEQ ID No. 42, SEQ ID No. 43, SEQ ID No. 44, SEQ ID No. 45, SEQ ID No. 46, SEQ ID No. 47, SEQ ID No. 48, SEQ ID No. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52 which result from a differential multiple splicing of the gene and/or of a different reading frame.

For example, the expression nucleic acid family encoding the saposin B protein family designates the nucleic acid family comprising *at least* the nucleic acids or fragments having the sequences SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55 which result from a

differential multiple splicing of the gene and/or of a different reading frame.

The expression "splicing" is understood to mean a mechanism of excision of the introns and of joining of the exons during the maturation of the transcripts and the expression "differential splicing" is understood to mean the existence of several schemes for splicing of a primary transcript resulting in the formation of different messenger RNAs and capable of leading to the synthesis of several different proteins (Kaplan and Delpech, Biologie Moléculaire et Médecine, 1993, 2nd edition, Médecine et Sciences, Flammarion, pages 73-77). This phenomenon is widely described in the scientific literature. By way of example, there may be mentioned the model of the genes which encode the heavy and light immunoglobulin chains, the model of the gene for dystrophin, the model of the gene for alpha-amylase, the gene for myelin, and the like.

It is known that the eukaryotic genes in particular comprise regions (exons) which encode fragments of the protein encoded by said gene and other regions (introns) which do not have a protein equivalent. This is due to the fact that the genes are first transcribed to a "primary" RNA which is then cut by splicing enzymes at the level of specific nucleotide sites (splicing sites). These enzymes then join the regions encoding the protein, thus reconstituting a "secondary" RNA from which the intron regions have been removed. Moreover, depending on the cellular phenotypes (and therefore the tissues or the differentiation), these enzymes are not all expressed, and thus the same RNA may be differently spliced in the cells of the same individual, thus generating proteins with differences in sequence. However, these phenomena may also be applied to nucleotide regions which are completely coding (exons), but which, according to different possible splicings, will generate several different

proteins from the same nucleotide region by the phenomenon of differential splicing between the different protein products.

Furthermore, it is known that nucleotide regions may have several reading frames according to the three potential frames of the genetic code. Thus, the presence of several initiation codons for translation in several reading frames and/or a splicing of primary RNA joining nucleotide sequences present in different reading frames on the DNA, allows the same DNA region to generate protein products with no mutual relationship from the point of view of the peptide sequence.

Finally, the genetic polymorphism existing between individuals of the same species and/or individual mutations can create or eliminate splicing sites from a given DNA region, and thus modify the sequence and the structure of the protein product(s) normally produced by this region.

Thus, the combination of these different phenomena can allow the same nucleotide sequence corresponding to a DNA segment, identified as determining a genetic region of interest in a given study, to comprise the information which is necessary and sufficient to define a whole family of RNA spliced according to different and alternative schemes, in various reading frames and, thereby obviously, proteins and polypeptides having "mosaic" sequences according to one reading frame or even according to the three potential frames and mutations possibly linked to genetic polymorphism.

An example of this phenomenon may be represented by the nucleotide region of the HIV-1 retrovirus *env* gene. Indeed, several different proteins are encoded by segments of the same sequence: for example, the

envelope glycoprotein, and the regulatory proteins TAT, REV, NEF, VIF.

It is also known that proteins may result from the assembly of identical subunits (homodimers, homomultimers) or different subunits (heterodimers, heteromultimers). Thus, the various protein products encoded by the same DNA region may also assemble with each other to constitute multimeric complex protein entities. This phenomenon is in addition to the preceding ones and, when a protein is identified by a peptide fragment, it is possible to logically identify all the other constituent elements of this complex protein and the spliced RNA and DNA segments encoding them, as well as all the members of the family of protein products and their assemblies. Another example is provided by the human DNA region encoding the family of MRP14, calgranulin B, MRP8, calprotectin and psoriasin proteins, and the like.

Accordingly, the subject of the present invention is the use of at least one polypeptide comprising at least one fragment of a protein to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease, said protein being chosen from proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any of the abovementioned

peptide sequences, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B. In specific embodiments, at least two abovementioned polypeptides are used in combination in order to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease.

The invention also relates to the use of at least one polypeptide comprising at least one fragment of a protein to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease, said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 8, SEQ ID No. 17 and SEQ ID No. 24 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the abovementioned peptide sequences. Advantageously, the five polypeptides which correspond to the above definition are used in combination.

Preferably, the peptide sequence of said polypeptide comprises, or consists of, a sequence chosen from any one of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 8, SEQ ID No. 17 and SEQ ID No. 24.

The invention also relates to the use of at least one fragment of one of the abovementioned polypeptides for the preparation of an immunogenic peptide, said peptide comprising all or part of at least one of the sequences

designated by the references SEQ ID Nos. 58 to 65 and being used for the production of monoclonal antibodies.

The subject of the invention is also the use of at least one nucleotide fragment to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease, according to which said nucleotide fragment is chosen from fragments which encode at least one fragment of a protein, said protein being chosen from proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% and advantageously at least 98% identity with any one of the above peptide sequences, and the fragments complementary to said fragments, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B. It is within the capability of persons skilled in the art to determine the nucleic sequences of the nucleotide fragments from the peptide sequences and the genetic code, this forming part of their general knowledge.

Preferably, said nucleotide fragment encodes a protein which, in the native state, consists of a sequence chosen from any one of the sequences SEQ ID Nos. 1 to 8 and SEQ ID Nos. 10 to 29 cited above, and among the peptide sequences or the fragments of said sequences

belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B.

Another subject of the invention is the use of at least one nucleotide fragment to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or neurological and/or autoimmune disease according to which said fragment is a fragment of a nucleic sequence chosen from any one of SEQ ID No. 30, SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 34, SEQ ID No. 35, SEQ ID No. 36, SEQ ID No. 37, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 40, SEQ ID No. 41, SEQ ID No. 42, SEQ ID No. 43, SEQ ID No. 44, SEQ ID No. 45, SEQ ID No. 46 and SEQ ID No. 47, SEQ ID No. 48, SEQ ID No. 49 and SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 67, SEQ ID No. 66, SEQ ID No. 69, SEQ ID No. 70 and SEQ ID No. 71, and their complementary sequences.

The invention also relates to the use of a ligand specific for a polypeptide or for a nucleotide fragment as defined above to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease.

The expression ligand is understood to mean any molecule capable of combining with a polypeptide, such as a monoclonal antibody, a polyclonal antibody, a receptor, a substrate with enzymatic activity, or an enzyme for which said polypeptide is a cofactor. The production of polyclonal or monoclonal antibodies forms part of the general knowledge of persons skilled in the

art. There may be mentioned, by way of reference, Köhler G. and Milstein C. (1975): Continuous culture of fused cells secreting antibody of predefined specificity, Nature 256:495-497 and Galfre G. et al. (1977) Nature, 266:522-550 for the production of monoclonal antibodies and Roda A., Bolelli G.F.: Production of high-titer antibody to bile acids, Journal of Steroid Biochemistry, Vol. 13, pp. 449-454 (1980) for the production of polyclonal antibodies.

The expression ligand is also understood to mean any molecule capable of combining with a nucleotide fragment, such as a partially or completely complementary nucleotide fragment, a complementary polynucleotide, or an anti-nucleic acid antibody. The production of nucleotide fragments or of polynucleotides forms part of the general knowledge of persons skilled in the art. There may be mentioned in particular the use of restriction enzymes, and chemical synthesis on an automated synthesizer, for example on synthesizers marketed by the company Applied Biosystem. Moreover, techniques for the production of anti-nucleic acid antibodies are known. There may be mentioned, by way of examples, Philippe Cros et al., Nucleic Acides Researc, 1994, Vol. 22, No. 15, 2951-2957; Anderson, W.F. et al. (1988) Bioessays, 8(2), 69-74; Lee, J.S. et al. (1984) FEBS Lett., 168, 303-306; Malfoy, B. et al. (1982) Biochemistry, 21(22), 5463-5467; Stollar, B.D. et al., J.J. (eds) Methods in Enzymology, Academic Press, pp. 70-85; Traincard, F. et al. (1989) J. Immunol. Meth., 123, 83-91 and Traincard, F. et al. (1989) Mol. Cell. Probes, 3, 27-38).

The subject of the invention is also a method for detecting at least one protein associated with a degenerative and/or autoimmune disease in a biological sample in which the biological sample is brought into contact with at least one ligand specific for at least one polypeptide, said polypeptide comprising at least

one fragment of a protein and said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to SEQ ID No. 8 and SEQ ID No. 10 to 29, and the peptide sequences or fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B, and then the formation of a complex between said polypeptide and said ligand is detected. Said ligand is advantageously a monoclonal antibody, a polyclonal antibody, a receptor, a substrate with enzymatic activity or an enzyme for which said polypeptide is a cofactor.

Likewise, the invention relates to a method for detecting at least one ligand associated with a degenerative and/or autoimmune disease, in a biological sample, characterized in that the biological sample is brought into contact with at least one polypeptide comprising at least one fragment of a protein, said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID

No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to SEQ ID No. 8 and SEQ ID Nos. 10 to SEQ ID No. 29, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B, and then the formation of a complex between said polypeptide and said ligand is detected. The ligand is any molecule which satisfies the conditions previously described.

Preferably, in the methods described above, the sequence of the polypeptide comprises or consists of a peptide sequence chosen from any one of SEQ ID No. 1 to 8 and SEQ ID No. 10 to 29 above and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B.

The invention also relates to a novel polypeptide which comprises at least one fragment of a protein whose peptide sequence corresponds to SEQ ID No. 9, said fragment exhibiting at least one mutation, in particular at least two mutations, in relation to the reference sequence SEQ ID No. 8. The polypeptide is advantageously chosen from the polypeptides which comprise the amino acid sequence FSWDNCFEGKDPAVIR, designated by the reference SEQ ID No. 68, and the amino acid sequence YSLPKSEFAVPDLELP, designated by the reference SEQ ID No. 72.

In particular, said polypeptide comprises or consists of SEQ ID No. 9. This polypeptide is used to obtain a diagnostic, prognostic, prophylactic or therapeutic

composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease, alone or as a mixture with at least one polypeptide as defined above.

One of the subjects of the invention is also a nucleotide fragment which encodes the fragment of the protein whose peptide sequence corresponds to SEQ ID No. 9, said fragment of said protein exhibiting at least one mutation, in particular two mutations relative to the reference sequence SEQ ID No. 8. Said nucleotide fragment in particular comprises or consists of a fragment which encodes SEQ ID No. 9. This fragment is used to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease, alone or as a mixture with at least one nucleotide fragment as defined above.

The subject of the invention is also a method for detecting at least one ligand associated with a degenerative and/or autoimmune disease, in a biological sample, according to which the biological sample is brought into contact with at least the polypeptide which comprises or consists of SEQ ID No. 9 or a mixture of polypeptides comprising this polypeptide and at least one polypeptide as described above, and then the formation of a complex or of complexes between the polypeptide(s) and the corresponding ligand(s) is detected; it is to be understood that the expression ligand is understood to mean a molecule which satisfies the abovementioned conditions.

The invention also relates to a method for detecting at least the reference polypeptide SEQ ID No. 9 or a fragment of said polypeptide, this fragment comprising at least one and preferably two mutations in relation to the reference sequence SEQ ID No. 8, in a biological

sample according to which the biological sample is brought into contact with at least one ligand specific for said polypeptide, and then the formation of a complex between said polypeptide and said ligand is detected. The definition of ligand corresponds to that defined above. It may be, inter alia, a monolonal antibody, a polyclonal antibody, a substrate with enzymatic activity or an enzyme for which said polypeptide is a cofactor, or a receptor.

It is also possible to bring the biological sample into contact with a ligand specific for the reference polypeptide SEQ ID No. 9 and at least one ligand specific for at least one other polypeptide as defined above, and then the formation of complexes between said polypeptides and said ligands specific for said polypeptides is detected; it being understood that the expression ligand is understood to mean a molecule which satisfies the conditions described above.

Another subject of the invention is a nucleotide fragment encoding all or part of the polypeptide SEQ ID No. 9, and its use to obtain a diagnostic, prognostic, prophylactic or therapeutic composition for detecting, prognosticating, preventing or treating a pathological condition associated with a degenerative and/or autoimmune disease, optionally in combination with at least one nucleotide fragment as defined above, and the fragments complementary to said fragments.

The expression polypeptide fragment is understood to mean at least all or part of the peptide sequence of a protein, in particular a polypeptide fragment which comprises between about 5 and 15 amino acids and more precisely between about 5 and 10 amino acids and 6 and 15 amino acids. The expression nucleotide fragment is understood to mean at least all or part of a nucleotide sequence, it being understood that the expression nucleotide sequence covers DNA and RNA sequences.

In particular, the expression polypeptide or nucleotide fragment is understood to mean either fragments associated with the same molecular unit, or fragments in a molecular complex comprising several homologous or heterologous subunits obtained naturally or artificially, in particular by differential multiple splicing or by selective synthesis.

The invention also relates to a method for detecting at least one polypeptide as defined above, according to which a sample of a biological fluid is collected from a patient having a pathological condition associated with a degenerative and/or neurological and/or autoimmune disease and, optionally after purification of said sample of biological fluid, the mass profile obtained from the biological fluid is analyzed by mass spectrometry and compared with a reference mass profile.

The present invention also relates to the use of *at least* one polypeptide of the invention to define therapeutically effective agents, and the use of these agents to prevent and/or treat an autoimmune and/or neurological and/or degenerative disease, and in particular multiple sclerosis.

Thus, other subjects of the invention are the following:

- Use of at least one polypeptide comprising at least one fragment of a protein to test the efficacy of a therapeutic agent, said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No.

18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29, the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B;

- Use of at least one polypeptide comprising at least one fragment of a protein to define a biological material for the preparation of a pharmaceutical composition for treating a degenerative and/or neurological and/or autoimmune disease, such as multiple sclerosis, said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29, the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin and saposin;

According to an advantageous variant of one of the preceding uses, the polypeptide is chosen from SEQ ID No. 2, 4, 8, 9, 17, 24;

- Use of at least one nucleotide fragment to test the efficacy of a therapeutic agent for a pathological condition associated with a degenerative and/or neurological and/or autoimmune disease, according to which said nucleotide fragment is chosen from the fragments which encode at least one fragment of a protein, said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29, the peptide sequences which exhibit at least 70% identity, preferably at least 80% and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and the fragments complementary to said fragments and the fragments which encode the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B.

- Use, to test the efficacy of a therapeutic agent for a pathological condition associated with a degenerative and/or neurological and/or autoimmune disease, of recombinant proteins and/or proteins encoded by all or part of the nucleotide fragments defined in the above paragraph;

- Use of at least one nucleotide fragment for the preparation of a pharmaceutical composition for treating a degenerative and/or neurological and/or autoimmune disease, such as multiple sclerosis, according to which said nucleotide fragment is chosen

from fragments which encode at least one fragment of a protein, said protein being chosen from the proteins whose peptide sequence in the native state corresponds to SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29, the peptide sequences which exhibit at least 70% identity, preferably at least 80% and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and the fragments complementary to said fragments and the fragments which encode the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B;

- Use, for the preparation of a pharmaceutical composition for treating a degenerative and/or neurological and/or autoimmune disease, such as multiple sclerosis, of recombinant proteins and/or proteins encoded by all or part of the nucleotide fragments defined in the preceding paragraph.

Advantageously, said nucleotide fragment used encodes said protein.

Preferably, the peptide sequence of said protein in the native state consists of a sequence chosen from any one of SEQ ID No. 1 to 29, the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins

chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B. The polypeptides are preferably chosen from SEQ ID No. 2, 4, 8, 9, 17, 24.

- Use of at least one nucleotide fragment to test the efficacy of a therapeutic agent for a pathological condition associated with a degenerative and/or neurological and/or autoimmune disease, according to which said fragment is a fragment of a nucleic sequence chosen from any one of SEQ ID No. 30, SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 34, SEQ ID No. 35, SEQ ID No. 36, SEQ ID No. 37, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 40, SEQ ID No. 41, SEQ ID No. 42, SEQ ID No. 43, SEQ ID No. 44, SEQ ID No. 45, SEQ ID No. 46 and SEQ ID No. 47, SEQ ID No. 48, SEQ ID No. 49 and SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No. 69, SEQ ID No. 70, SEQ ID No. 71, and their complementary sequences.

- Use of at least one nucleotide fragment for the preparation of a pharmaceutical composition for treating a degenerative and/or neurological and/or autoimmune disease, such as multiple sclerosis, according to which said fragment is a fragment of a nucleic sequence chosen from any one of SEQ ID No. 30, SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 34, SEQ ID No. 35, SEQ ID No. 36, SEQ ID No. 37, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 40, SEQ ID No. 41, SEQ ID No. 42, SEQ ID No. 43, SEQ ID No. 44, SEQ ID No. 45, SEQ ID No. 46 and SEQ ID No. 47, SEQ ID No. 48, SEQ ID No. 49 and SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No 68, SEQ ID No. 69, SEQ ID No. 70, SEQ ID No. 71, and their complementary sequences.

The nucleic sequence is preferably chosen from SEQ ID No. 30, 31, 42, 53.

- Use of lycorine for the preparation of a composition for preventing and/or treating a degenerative and/or neurological and/or autoimmune disease.

The expression therapeutic efficacy is understood to mean the clinical and biological benefit acquired after administration of a therapeutic agent for the purpose of improving or even curing the disease. This benefit is manifested, inter alia, by a reduction in the clinical and biological signs, and in the pathological effects of the disease after clinical analysis by the doctor and/or biological analyses, such as magnetic resonance imaging, analysis of the oligoclonal bands in the cerebrospinal fluid, analysis of evoked potentials and the test for detection of gliotoxicity called bioassay, whose principle is described in patent application WO 98/11439 cited above. This reduction in the clinical signs and pathological effects should result in a benefit for the patient (Schwartz and Lazar, 1995, Elements de statistique médicale et biologique, eds Flammarion; Lazar and Schwartz, 1995, Eléments de statistique médicale et biologique, eds Flammarion). The disease studied is preferably multiple sclerosis.

The expression composition for prophylactic and/or therapeutic use is understood to mean any composition which comprises an effective therapeutic agent. These therapeutic agents are capable (i) of qualitatively and/or quantitatively influencing the biological activity and/or the function of the proteins of interest identified in the present invention, preferably the gliotoxic activity and/or (ii) modulating and/or inhibiting the expression of these proteins and/or (iii) reducing the concentration of

these proteins in an extracellular and/or intracellular compartment, and/or substituting a nonpathogenic form for a pathogenic, for example mutated, form of one of these proteins and/or modulating their attachment to at least one of their ligands; said ligand being a molecule which satisfies the criteria described above. Various therapeutic agents are produced based on the conventional approaches widely described in the literature. The various groups of therapeutic agents defined from the proteins of interest identified in this present invention are described below. Their prophylactic and/or therapeutic efficacy or activity is evaluated *in vitro* and/or *in vivo*.

Evaluation of the efficacy of a therapeutic agent *in vitro*: urine samples from healthy individuals and from patients suffering from multiple sclerosis, preferably in the active phase, are tested for their gliotoxic activity *in vitro* based on the bioassay protocol described in patent application WO 98/11439, cited above. The experiment is carried out in parallel by adding or otherwise, to the urine samples tested, the therapeutic agent whose efficacy is to be tested. Assays are carried out at various concentrations of this agent, and after various incubation times with the sample, at a temperature of about 37°C or at room temperature, for each concentration of agent tested, before carrying out the bioassay test. The gliotoxic activity is determined for each crude or purified sample of control and patient's urine in the presence or in the absence of tested therapeutic agent. A prophylactic and/or therapeutic agent for multiple sclerosis is an agent which allows a reduction or an inhibition of the gliotoxic activity in a biological fluid from the patients, in particular in the urine. This reduction or inhibition is evaluated relative to the gliotoxic activity detected in the biological fluid of MS patients in the absence of the test agent which defines the upper limit and relative to the gliotoxic

activity detected in the urine of a healthy individual which determines the lower limit (Schwartz and Lazar, 1995, Elements de statistique médicale et biologique, eds Flammarion; Lazar and Schwartz, 1995, Elements de statistique médicale et biologique, eds Flammarion). The therapeutic efficacy of several agents may be evaluated in combination in the same assay.

Evaluation of the efficacy of a therapeutic agent using an animal model: there are injected into an animal fractions of purified urine and/or at least one polypeptide of the invention and/or at least one protein obtained by genetic recombination which corresponds to at least one polypeptide of the invention and/or at least one synthetic polypeptide whose amino acid sequence corresponds to the sequence of at least one polypeptide of the invention. The injections are carried out, at various established concentrations, into mammalian animals such as mice or rats, preferably a Lewis rat according to the protocol described in patent application WO97/33466 cited above. Various concentrations of a fraction of crude or purified urine or of at least one polypeptide and/or one protein, as defined above, are injected into a series of animals by the intradermal, intravenous, intrathecal, intracerebral or intramuscular route, and the like. A negative control is carried out in parallel. The prophylactic and/or therapeutic agent to be evaluated and then injected at various concentrations and by various routes of administration to a mammalian animal, preferably to a mouse or to a rat. The injections are carried out as a single dose or as repeated doses, with various time intervals between each administration. A few hours to a few weeks after the administration, biological samples, preferably of blood, serum, cerebrospinal fluid, or urine, are collected. These samples are subjected to:

(i) a measurement of the gliotoxic activity by the bioassay, and/or

(ii) a measurement of activity of the polypeptides and/or proteins of interest of the invention, alone or in combination, as described at least in: Li et al., 1983, Am J Hum Genet 35:629-634; Li et al., 1988 J Biol Chem 263:6588-6591; Li et al., 1981 J Biol Chem 256: 6234-6240; Li et al., 1976 J Biol Chem 251:1159; Kase et al., 1996, FebsLetters 393: 74-76; Kishimoto et al., 1992, J Lipid Res 33: 1255-1267; O'Brien et al., 1991 Faseb J 5: 301-308; Murthy et al., 1993 J Immunol 151: 6291-6301; Murao et al., 1990 Cell growth Differ 1: 447-454, and/or

(iii) an assay of the polypeptides and/or proteins of interest, alone or in combination, by ELISA (Enzyme Linked-Immunosorbant Assay) and/or Western blotting, using antibodies or antibody fragments capable of binding to at least one of the polypeptides and/or proteins of the invention, or their fragment, and/or

(iv) an assay of antibodies specific for the polypeptides and/or proteins of interest or their fragments, alone or in combination or the assay of at least one ligand capable of binding to the polypeptides and/or proteins of interest or their fragments, and/or

(v) an assay of the "helper" and/or cytotoxic cellular immune response induced against the polypeptides and proteins of interest or their fragments and any immunogenic peptide derived from these polypeptides, proteins and fragments, by carrying out, for example, a test of activation *in vitro* of "helper" T lymphocyte cells specific for the antigen administered; by quantifying the cytotoxic T lymphocytes according to the so-called ELISPOT technique described by Scheibenbogen et al., 1997 Clinical Cancer Research 3: 221-226. Such a determination is particularly advantageous when it is desired to evaluate the efficacy of a vaccine approach for use in a given patient or for diagnosing and/or prognosticating a potential pathological condition by seeking to demonstrate an immune response naturally developed by the patient against the antigen, the polypeptides, the

proteins of interest or the immunogenic fragments derived from these proteins.

The expression "ligand capable of binding to a protein" is understood to mean any molecule capable of recognizing the protein or a portion of the protein. This may be verified for example *in vitro* by Elisa and/or Western blot tests.

The expression "polypeptides and/or proteins of interest of the invention" designates the C-terminal fragment of Perlecan (SEQ ID No. 2), the precursor of the retinol-binding plasma protein (SEQ ID No. 4), the GM2 activator protein (SEQ ID No. 8), the mutated protein of the GM2 activator (SEQ ID No. 9), calgranulin B (SEQ ID No. 17), saposin B (SEQ ID No. 24), the proteins or fragments belonging to the family of the precursor of the retinol-binding plasma protein (for example SEQ ID No. 5 to 7), the proteins or fragments belonging to the family of the GM2 activator protein (for example SEQ ID No. 10 to 16), the proteins or fragments belonging to the family of calgranulin B protein (for example SEQ ID No. 18 to 23), the proteins or fragments belonging to the family of the saposin B protein (for example SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29.

The animal is then sacrificed and histological sections of various tissues are prepared, preferably brain sections. Various studies and observations are carried out in order to detect and/or quantify the characteristic effects of the polypeptides and/or active proteins associated with the gliotoxic fraction, that is to say an apoptosis of the glial cells, and/or the opening of the blood-brain barrier and/or a demyelination. The presence or the expression of the

polypeptides and/or proteins of interest identified is also observed and/or quantified in these tissues:

(i) by conventional immunohistological analyses using ligands for the polypeptides and/or proteins of interest and/or their fragments and/or monoclonal or polyclonal antibodies or fragments of said which bind to the polypeptides and/or proteins of interest, or to their fragments, and/or

(ii) by conventional *in situ* hybridization techniques using nucleic acid fragments or oligonucleotides defined from polypeptide and/or protein sequences of interest; and/or

(iii) by PCR and/or RT-PCR amplification techniques in situ using nucleic acid fragments or primers defined from polypeptide and/or protein sequences of interest.

The expression antibodies capable of binding to a polypeptide, to a protein or to their fragments is understood to mean any monoclonal or polyclonal antibody and any fragment of said antibodies capable of recognizing the polypeptide, the protein or their fragments. The capacity of the antibodies to recognize said polypeptides, proteins or their fragments is verified *in vitro*, for example by ELISA and/or Western blotting. An antibody capable of binding to the saposin B protein (SEQ ID No. 24) or to any fragment of this protein is described by Misasi et al. 1998, J. NeuroChem. 71:2313 and Klein et al. 1994, BBRC 200: 1440-1448 or may be produced using conventional methods, for example those designated by references above for the production of monoclonal and polyclonal antibodies, by immunization starting with a natural protein, a recombinant protein, a synthetic polypeptide or their fragments. The immunogenic peptides for the production of anti-saposin B monoclonal antibodies are the peptides corresponding to the sequences SEQ ID No. 61 and SEQ ID No. 62.

For example, an antibody capable of binding to the GM2 activator protein (SEQ ID No. 8) or to any fragment of this protein is illustrated by Yuziuk et al., 1998 J Biol Chem 273: 66-72 or may be produced using conventional methods known to persons skilled in the art. This antibody may for example be produced after injecting into mice or rabbits the natural protein or any fragment, and/or the recombinant protein or any fragment, and/or peptides defined and synthesized from the protein sequence of the protein. The immunogenic peptides used for the production of anti-GM2 monoclonal antibodies are the reference peptides SEQ ID No. 58, SEQ ID No. 59 and SEQ ID No. 60. An antibody capable of binding to the galgranulin B protein (SEQ ID No. 17) or to any fragment of this protein is described by Saintigny et al., 1992 J Invest Dermatol 99: 639-644 and Goebeler et al. 1994 J Leukoc Biol 55: 259-261, or may be produced using conventional methods. The immunogenic peptides for the production of anti-calgranulin B monoclonal antibodies are the peptides corresponding to the sequences SEQ ID No. 63, SEQ ID No. 64 and SEQ ID No. 65. An antibody capable of binding to the mutated GM2 activator protein (SEQ ID No. 9) or to any fragment of this protein may be produced using the conventional methods defined above.

The expression natural protein and fragment is understood to mean any isolated, completely or partially purified protein obtained from a human or animal sample and any fragment obtained from this protein. For example, the natural protein corresponding to saposin B (SEQ ID No. 24) is obtained according to the technique described by Waring et al. 1998 Mol Genet Metab 63: 14-25; the natural protein corresponding to the GM2 activator protein (SEQ ID No. 8) according to the technique described by DeGasperi et al., 1989 Biochem J 260: 777-783, Vogel et al., 1987 Arch Biochem Biophys 259: 627-638, Mitsuyama, 1983 Hokkaido Igaku Zasshi 58: 502-512; Hirabayashi et al 1983 J Neurochem

40: 168-175, Conzelmann et al., 1979 Hoppe Seylers Z Physiol Chem 360: 1837-1849, Li et al., 1976 J Biol Chem 251: 1159-1163. The natural protein corresponding to calgranulin B (SEQ ID No. 17) is obtained according to the technique described by Hitomi et al., 1996 J Cell Sci 109: 805-815, Van den Bos et al. 1998 Protein Expr Purif 13: 313-318 and Raftery et al. 1996 Biochem J 316: 285-293.

The expression recombinant protein or fragment of a recombinant protein refers to any protein or protein fragment produced in a prokaryotic or eukaryotic cell from a nucleotide sequence encoding the protein or its fragment and transfected into the cell, this protein or its fragment then being purified. In general, any cell derived from a prokaryotic or eukaryotic organism may be used in the context of the present invention, but the cells derived from eukaryotic organisms are preferred. There may be mentioned, by way of example, CHO cells, COS cells, and Semliki cells. For the purposes of the present invention, said cell may be wild type or mutant. For example, the recombinant protein corresponding to saposin B (SEQ ID No. 24) may be obtained according to the techniques described by Zaltash et al. 1998 Bebbs letter 423: 1-4 and Qi et al. 1994 J Biol Chem 269: 16746-16753. Such a recombinant protein is at least available from Kase et al. 1996 Febs Lett 393: 74-76. The recombinant protein corresponding to the GM2 activator protein (SEQ ID No. 8) may be produced by the techniques described by Yuziuk et al. 1998 J Biol Chem 273: 66-72 and Bierfreund et al., 1999 Neurochem Res 24: 295-300. The recombinant protein corresponding to calgranulin B (SEQ ID No. 17) may be obtained according to the protocol by Longbottom et al. 1992 Biochim Biophys Acta 1120:215-222, Raftery et al. 1999 Protein Expr Purif 15:228-235. Such a recombinant protein is available at least from Klempt et al. 1997 Febs Letter 408:81-84.

The expression DNA nucleotide sequence or DNA nucleotide fragment encoding all or part of the saposin B protein (SEQ ID No. 24) is understood to mean the nucleic acid sequence SEQ ID No. 53 or a fragment of this sequence. The expression RNA nucleotide sequence or fragment encoding all or part of the saposin B protein (SEQ ID No. 24) is understood to mean any sequence deduced from the DNA sequence SEQ ID No. 53, taking into account the genetic code and the splicing phenomena.

The expression DNA nucleotide sequence or DNA nucleotide fragment encoding all or part of the GM2 activator protein (SEQ ID No. 8) is understood to mean the nucleic acid sequence SEQ ID No. 31 or a fragment of this sequence. The expression RNA nucleotide sequence or fragment encoding all or part of the GM2 activator protein (SEQ ID No. 8) is understood to mean any sequence deduced from the DNA sequence SEQ ID No. 31, taking into account the genetic code and the splicing phenomena.

The expression DNA nucleotide sequence or DNA nucleotide fragment encoding all or part of the calgranulin B protein (SEQ ID No. 17) is understood to mean the nucleic acid sequence SEQ ID No. 42 or a fragment of this sequence. The expression RNA nucleotide sequence or fragment encoding all or part of the calgranulin B protein (SEQ ID No. 17) is understood to mean any sequence deduced from the DNA sequence SEQ ID No. 42, taking into account the genetic code and the splicing phenomena.

The expression nucleotide sequence or fragment encoding all or part of the mutated protein (SEQ ID No. 9) is understood to mean the nucleic acid sequence deduced from the sequence SEQ ID No. 9, taking into account the genetic code. The expression RNA nucleotide sequence or fragment encoding all or part of this mutated B protein

(SEQ ID No. 9) is understood to mean any sequence deduced from the DNA sequence, taking into account the genetic code and the splicing phenomena.

The expression protein activity is understood to mean a characteristic biological function of the protein. The protein activity may be demonstrated by techniques known to persons skilled in the art. For example, the activity of saposin B (SEQ ID No. 24) and of the proteins of the saposin B family (for example SEQ ID No. 25 to 29) may be detected using the protocols described by Li et al., 1983, Am J Hum Genet 35:629-634; Li et al., 1988 J Biol Chem 263: 6588-6591, Li et al., 1981 J Biol Chem 256: 6234-6240 and Li et al., 1976 J Biol Chem 251:1159. The expression activity of the GM2 activator protein (SEQ ID No. 8) and of the proteins of the same family (for example SEQ ID No. 10 to 16) is understood to mean at least the activity detected using the protocols described, for example, by Kase et al., 1996, Febs Letters 393: 74-76, Kishimoto et al., 1992, J Lipid Res 33:1255-1267 and O'Brien et al., 1991 Faseb J 5: 301-308. The expression activity of calgranulin B (SEQ ID No. 17) and the proteins of the same calgranulin B family (for example SEQ ID No. 18 to 23) and any is understood to mean at least the activity detected using the protocols described for example by Murthy et al., 1993 J Immunol 151: 6291-6301 and Murao et al., 1990 Cell growth Differ 1: 447-454.

Production of a transgenic animal, preferably murine, model for a human pathology can be technically achieved. Briefly, the transgenic animal is produced using the conventional techniques described and possesses, integrated into the genome, the nucleic acids encoding the proteins or their fragments.

Evaluation of the efficacy of a therapeutic agent and therapeutic monitoring *ex vivo*, in humans:

the therapeutic agents to be tested for a therapeutic activity and/or for therapeutic monitoring are administered by various routes to humans, such as the intradermal, intravenous, intramuscular, intracerebral or oral routes, and the like. Various doses are administered to human beings. The patient's clinical file at the time of the first administration is perfectly known. One or more administrations may be carried out with various time intervals between each administration which may range from a few days to a few years. Biological samples are collected at defined time intervals after administration of the therapeutic agent, preferably blood, serum, cerebrospinal fluid and urine. Various analyses are carried out using these samples. Immediately before the first administration of the therapeutic agent, these sample collections and these same analyses are again performed. A conventional clinical and biological examination (MRI, oligoclonal bands in cerebrospinal fluid, evoked potentials) is also carried out in parallel with the additional analyses which are described below, at various analytical times. The analyses carried out are:

(i) a measurement of the gliotoxic activity by bioassay starting with samples of serum, CSF and urine, and/or
(ii) a measurement of the activity of proteins of interest identified in the present invention alone or in combination, as described for example by: Li et al., 1983, Am J Hum Genet 35:629-634; Li et al., 1988 J Biol Chem 263: 6588-6591; Li et al., 1981 J Biol Chem 256: 6234-6240; Li et al., 1976 J Biol Chem 251:1159; Kase et al., 1996, FebsLetters 393:74-76; Kishimoto et al., 1992, J Lipid Res 33: 1255-1267; O'Brien et al., 1991 Faseb J 5: 301-308; Murthy et al., 1993 J Immunol 151: 6291-6301; Murao et al., 1990 Cell growth Differ 1: 447-454; and/or
(iii) an assay of the proteins of interest or of their fragments, alone or in combination, in the blood/ serum, CSF or urine samples by ELISA and/or Western

blotting, using antibodies or antibody fragments capable of binding to at least one of the proteins or to one of their fragments, and/or

(iv) an assay of antibodies specific for the proteins of interest or of their fragments in blood/serum, CSF or urine samples, by ELISA and/or Western blotting using a natural protein or a fragment of the natural protein and/or a recombinant protein or a fragment of this recombinant protein, alone or in combination. Likewise, an assay of ligands capable of binding to the proteins of interest identified, alone or in combination, may be carried out, and/or

(v) an assay of "helper" and/or cytotoxic cellular immune response induced against the proteins of interest and any immunogenic peptide derived from these proteins, for example by carrying out a test of activation *in vitro* of T lymphocyte cells specific for the antigen administered (example). For example, using a test of activation *in vitro* of helper T lymphocyte cells specific for the antigen administered (example); For example by quantifying the cytotoxic T lymphocytes according to the so-called ELISPOT technique described by Scheibenbogen et al., 1997 Clinical Cancer Research 3: 221-226. Such a determination is particularly advantageous when it is desired to evaluate the efficacy of a vaccine approach used in a given patient or to diagnose a potential pathological condition in a patient, seeking to demonstrate an immune response naturally developed by said patient against the antigen, the proteins of interest or any immunogenic fragment derived from these proteins, alone or in combination, and/or

(vi) a detection of DNA and/or RNA fragments encoding the proteins or a fragment of proteins of interest by nucleotide hybridization according to techniques well known to persons skilled in the art (Southern blotting, Northern blotting, ELOSA "Enzyme-linked Oligosorbent Assay" (Katz JB et al., Am. J. Vet. Res., 1993 Dec; 54 (12):2021-6 and Francois Mallet et al., Journal of

Clinical Microbiology, June 1993, p1444-1449)) and/or by the DNA and/or RNA amplification method, for example by PCR, RT-PCR, using nucleic acid fragments encoding the sequence of the proteins of interest, and/or

(vii) by tissue, preferably brain, biopsy and observation of the characteristic effects of the active proteins associated with the gliotoxic fraction, that is to say an apoptosis of the glial cells and/or the opening of the blood-brain barrier and/or the observation of demyelination phenomena, and/or

(viii) by tissue biopsy or on circulating cells (blood, CSF), observation of the presence of proteins of interest and estimation of their expression by immunohistological observation on histological sections prepared from tissues, using ligands and/or antibodies or their fragments capable of binding to the proteins of interest, and/or

(ix) by tissue biopsy or on circulating cells (blood, CSF), observation of the expression of the proteins of interest by in situ hybridization of the RNA molecules encoding the proteins of interest using nucleic acids defined using the sequences of the proteins of interest, and/or

(x) by tissue biopsy or on circulating cells (blood, CSF), determination of the expression of the proteins of interest by amplification of these RNAs by conventional techniques such as, for example, RT-PCR, using nucleic acids defined using the sequences of the proteins of interest.

The expression "polypeptides and/or proteins of interest of the invention" designates the C-terminal fragment of Perlecan (SEQ ID No. 2), the precursor of the retinol-binding plasma protein (SEQ ID No. 4), the GM2 activator protein (SEQ ID No. 8), the mutated GM2 activator protein (SEQ ID No. 9), calgranulin B (SEQ ID No. 17), saposin B (SEQ ID No. 24), the proteins or fragments belonging to the family of the precursor of the retinol-binding plasma protein (for example SEQ ID

No. 5 to 7), the proteins or fragments belonging to the family of the GM2 activator protein (for example SEQ ID No. 10 to 16), the proteins or fragments belonging to the calgranulin B protein family (for example SEQ ID No. 18 to 23), the proteins or fragments belonging to the saposin B protein family (for example SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29.

The expression DNA nucleic acid sequence or fragments encoding the "polypeptides and/or proteins of interest of the invention" designates the nucleic acid sequence encoding the C-terminal fragment of Perlecan (SEQ ID No. 2), the nucleic acid sequence encoding the precursor of the retinol-binding plasma protein (SEQ ID No. 4), the nucleic acid sequence (SEQ ID No. 31) encoding the GM2 activator protein (SEQ ID No. 8), the nucleic acid sequence encoding the mutated GM2 activator protein (SEQ ID No. 9), the nucleic acid sequence (SEQ ID No. 42) encoding calgranulin B (SEQ ID No. 17), the nucleic acid sequence (SEQ ID No. 53) encoding saposin B (SEQ ID No. 24), the DNA and RNA nucleic acid sequences (SEQ ID No. 30 to 57) encoding the proteins or fragments belonging to the family of the precursor of the retinol-binding plasma protein (for example SEQ ID No. 5 to 7), the proteins or fragments belonging to the family of the GM2 activator protein (for example SEQ ID No. 10 to 16), the proteins or fragments belonging to the calgranulin B protein family (for example SEQ ID No. 18 to 23), the proteins or fragments belonging to the saposin B protein family (for example SEQ ID No. 25 to 29).

A protein or a variant of a protein chosen more particularly from the sequences defined in the identifiers SEQ ID Nos. 2, 4, 8, 9, 17 and 24 or their fragments, or from the sequences corresponding to the proteins of the families of said sequences (SEQ ID No.

1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 24, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, independently or in combination, exhibits a toxic effect directly or indirectly on cells, in particular on glial cells, which is demonstrated by the abovementioned bioassay. The autoantibodies produced in response to the presence of this protein or of these proteins are associated with the autoimmune process. Thus, the target of the therapeutic agent(s) is for example (i) the natural protein or the natural proteins or their variants with the aim of regulating their expression and/or their intracellular concentration and/or their concentration in the bloodstream, (ii) an antibody specific for at least such a protein. The therapeutic agent or the therapeutic agents defined eliminate the target directly, by inducing a specific immune response, and/or neutralize it.

The present invention therefore relates to a biological material for the preparation of a pharmaceutical composition for treating mammals suffering from degenerative and/or autoimmune and/or neurological pathological conditions, preferably multiple sclerosis, said composition comprising:

(i) either at least one natural protein and/or one recombinant protein or their fragments whose sequence corresponds to all or part of the sequences designated by the references SEQ ID No. 2, 4, 8, 9, 17 and 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16,

SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, independently or in combination,

(ii) or at least one ligand specific for at least one of said proteins or their fragments whose sequence corresponds to all or part of the sequences designated by the references SEQ ID No. 2, 4, 8, 9, 17 and 24, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, independently or in combination,

(iii) or at least one polyclonal or monoclonal antibody specific for at least one of said proteins or their fragments whose sequence corresponds to all or part of the sequences designated by the references SEQ ID No. 2, 4, 8, 9, 17 and 24, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, independently or in combination,

(iv) or at least one nucleic acid sequence comprising at least one gene of therapeutic interest whose nucleic sequence is deduced from the DNA and RNA sequences encoding all or part of the proteins whose sequences are designated by the references SEQ ID No. 2, 4, 8, 9, 17 and 24, and the DNA and/or RNA sequences (for example SEQ ID No. 30 to 57) encoding all or part of the proteins belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B, in association with elements ensuring the expression of said gene of therapeutic interest *in vivo* in target cells intended to be genetically modified by the nucleic sequence of the gene of therapeutic interest,

(v) or at least one mammalian cell not naturally producing the protein of interest or the proteins of interest or any fragment of this or these protein(s) or of the antibodies specific for at least one of said proteins or of its fragments, said mammalian cell being genetically modified *in vitro* by at least one nucleic acid sequence or a fragment of a nucleic acid sequence or a combination of nucleic acid sequences corresponding to nucleic acid fragments derived from the same gene or from different genes, said nucleic sequence(s) being deduced from the DNA and RNA sequences encoding the proteins designated by the references SEQ ID No. 2, 4, 8, 9, 17 and 24, and the DNA and/or RNA sequences (for example SEQ ID No. 30 to 57) encoding all or part of the proteins belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B, said gene of therapeutic interest encoding all or part of the protein of interest, of a fragment of the protein of interest or of an antibody specific for the protein of interest which will be expressed at the surface of said mammalian cell (Toes et al., 1997, PNAS 94: 14660-

14665). The pharmaceutical composition may contain a therapeutic agent alone directed against a target alone or agents taken in combination directed against several targets.

The expression "polypeptides and/or proteins of interest of the invention" designates the C-terminal fragment of Perlecan (SEQ ID No. 2), the precursor of the retinol-binding plasma protein (SEQ ID No. 4), the GM2 activator protein (SEQ ID No. 8), the mutated GM2 activator protein (SEQ ID No. 9), calgranulin B (SEQ ID No. 17), saposin B (SEQ ID No. 24), the proteins or fragments belonging to the family of the precursor of the retinol-binding plasma protein (for example SEQ ID No. 5 to 7), the proteins or fragments belonging to the family of the GM2 activator protein (for example SEQ ID No. 10 to 16), the proteins or fragments belonging to the calgranulin B protein family (for example SEQ ID No. 18 to 23), the proteins or fragments belonging to the saposin B protein family (for example SEQ ID No. 25 to 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29.

From the knowledge of the amino acid sequences of the proteins of interest identified in the present invention, it is within the capability of persons skilled in the art to define and use the molecules described above and/or any molecule capable of binding to said molecules, and/or any molecule capable of inhibiting said molecules. Thus, the present invention relates to the use of natural and/or recombinant proteins and/or of synthetic polypeptides and their fragments, of ligands capable of binding to said proteins or to their fragment(s), for example antibodies; proteins inhibiting the function and/or expression and/or binding of said proteins.

Use of natural protein(s) and/or peptide(s) and/or recombinant protein(s) and/or synthetic polypeptide(s) corresponding to the proteins of interest identified in the present invention.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for treating mammals suffering from an autoimmune disease, preferably multiple sclerosis, comprising:

(i) either at least one natural protein and/or one recombinant protein and/or one synthetic polypeptide chosen from the proteins whose amino acid sequences are designated by the references SEQ ID No. 2, 4, 8, 9, 17 and 24, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, alone or in combination,

(ii) or at least one natural and/or synthetic fragment of these proteins of interest, for example an immunogenic fragment capable of inducing an immune response against a target polypeptide,

(iii) or at least one mimotope peptide defined from the reference sequences SEQ ID No. 2, 4, 8, 9, 17 and 24, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16,

SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, or a combination of mimotopes, capable of inducing an immune response against the target polypeptide,

(iv) or at least any protein or peptide capable of regulating *in vivo* the transcription and/or the translation of the proteins of interest (SEQ ID No. 2, 4, 8, 9, 17 and 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7,, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29. The administration of these proteins and/or peptides alone or in combination can reestablish the concentration of a protein of interest in the body.

The immune response directed against a specific antigen may be divided into two distinct categories, one involving the antibodies (humoral type immune response), the other the cytotoxic effector cells such as for example the macrophages, the cytotoxic lymphocytes (CTL) or the killer (NK) cells as well as the "helper" T lymphocytes, in particular the CD4+ T lymphocytes (cellular type immune response). More particularly, the two types of response are distinguishable in that the antibodies recognize the antigens under their three-dimensional form whereas the T lymphocytes, for example, recognize peptide portions of said antigens, associated with glycoproteins encoded

by the genes of the major histocompatibility complex (MHC), in particular the genes of the type I major histocompatibility complex which are ubiquitously expressed at the surface of the cells or the genes of the type II major histocompatibility complex which are specifically expressed at the surface of the cells involved in the presentation of antigens (APC). 1) According to a first aspect, the cellular type immune response is characterized in that the CD4+ type T cells (helper T cells), following a well-known activation phenomenon (for a review see Alberolalia 1997, Annu Rev Immunol 15, 125-154), produce cytokines which in turn induce the proliferation of APC cells capable of producing said cytokines, the cellular differentiation of the B lymphocytes capable of producing antibodies specific for the antigen, and the stimulation of the cytotoxic T lymphocytes (CTL). 2) According to a second aspect of the cellular immune response, the cytotoxic effector cells such as for example the CD8+ type lymphocytes (CTL) are activated a) after interaction with antigenic peptides bound to and presented by the glycoproteins carried by the ubiquitous cells and encoded by the genes belonging to the MHCI system, and b) optionally by the cytokines produced by the CD4+ cells.

The present invention relates to the administration of a protein or of a peptide derived from the proteins of interest (SEQ ID No. 2, 4, 8, 9, 17 and 24) or of their fragment(s), and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide

sequences SEQ ID No. 1 to 29, alone or in combination, for the prophylaxy and/or the therapy of an autoimmune disease, such as multiple sclerosis. These administered proteins and peptides are characterized in that they must have lost their toxic activity, for example their gliotoxic activity, or must have lost their capacity to bind to a ligand, and may significantly induce an immune response mediated by the T lymphocytes and/or the antibodies directed against this protein are used. Such proteins are said to be "modified"; nevertheless, their immunogenicity is preserved. Such modified immunogenic molecules are obtained by a number of conventional treatments, for example chemical or heat denaturation, truncation or mutation with deletion, insertion or location of amino acids. An example of truncation consists in the truncation of amino acids at the carboxy-terminal end which may be up to 5-30 amino acids. The modified molecules may be obtained by synthetic and/or recombinant techniques or by chemical or physical treatments of the natural molecules.

The natural and/or recombinant proteins of interest identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17 and 25), and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98 identity with any one of the peptide sequences SEQ ID No. 1 to 29, or their fragment(s), are used in prophylactic and therapeutic vaccination against autoimmune diseases, preferably MS. A vaccine comprises an immunogenically effective quantity of the immunogenic protein in association with a pharmaceutically acceptable vehicle and optionally an

adjuvant and/or a diluent. The pharmaceutically acceptable vehicles, adjuvants and diluents are well known to persons skilled in the art. There may be mentioned, by way of references, Remington's Pharmaceutical Sciences. The use of vaccine compositions is particularly advantageous in association with an early diagnosis of the disease. The immunogenic protein is used in the preparation of a medicament for prophylactic or therapeutic vaccination. The proteins of interest may be eliminated from the body without inducing undesirable side effects. The identification of such vaccine proteins or peptides is carried out as follows: the candidate molecules modified as described above (proteins which are natural or recombinant, peptides) are analyzed in a functional test to verify that they have lost their toxicity, for example their gliotoxic activity, using the test known as bioassay, and to verify their immunogenicity (i) by carrying out an *in vitro* test of proliferation of CD4+ T lymphocytes specific for the antigen administered (T cell assay) or an *in vitro* test of cytotoxicity of the CD8+ lymphocytes specific for the antigen administered and (ii) by measuring, inter alia, the amount of circulating antibodies directed against the natural protein. These modified forms are used to immunize humans by standard procedures with appropriate adjuvants.

The prepared vaccines are injectable, that is to say in liquid solution or in suspension. Optionally, the preparation may also be emulsified. The antigenic molecule may be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Examples of favorable excipients are water, saline solution, dextrose, glycerol, ethanol or equivalents and their combinations. If desired, the vaccine may contain minor quantities of auxiliary substances such as "wetting" or emulsifying agents, pH buffering agents or adjuvants such as aluminum

hydroxide, muramyl dipeptide or variations thereof. In the case of peptides, their coupling to a larger molecule (KLH, tetanus toxin) sometimes increases the immunogenicity. The vaccines are conventionally administered by injection, for example by subcutaneous or intramuscular injection. Additional formulations favorable with other modes of administration include suppositories and sometimes oral formulations.

In general, the concentration of the polynucleotide in the composition used for administration *in vivo* is from 0.1 µg/ml up to 20 mg/ml. The polynucleotide may be homologous or heterologous for the target cell into which it will be introduced.

The present invention also relates to the use of vaccines including molecules of nucleic acids which encode the proteins of interest or immunogenic peptides or their fragment(s), which are non-active, corresponding to the proteins of interest (SEQ ID No. 2, 4, 8, 9, 17 and 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29. The nucleic acid vaccines, in particular the DNA vaccines, are generally administered in association with a pharmaceutically acceptable vehicle by intramuscular injection.

From the amino acid sequence of the proteins of interest described (SEQ ID No. 2, 4, 8, 9, 17 and 24) and the peptide sequences or the fragments of said

sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, peptides or fragments corresponding to all or part of the primary sequence of these proteins may be synthesized by conventional methods of peptide synthesis or obtained by genetic recombination.

Recombinant proteins corresponding to the proteins of interest, produced in a prokaryotic or eukaryotic cellular system, are available from various teams and are described in the literature. They may also be produced by persons skilled in the art from the knowledge of the sequences of the corresponding genes described in the literature and taking into account the degeneracy of the genetic code. All the protein sequences identified in the present invention are thus capable of being obtained by genetic recombination. The genes are cloned into suitable vectors. Different vectors are used to transform prokaryotic cells (for example *E. coli*) and eukaryotic cells (for example COS cells, CHO cells and Simliki cells). The recombinant proteins corresponding to the proteins of interest or to fragments of the proteins of interest may thus be produced in prokaryotic and/or eukaryotic cellular systems. In *E. coli* cells, the recombinant proteins are produced with a polyhistidine tail. The insoluble protein fraction is solubilized in 8M urea. Enrichment of the product was carried out on nickel-chelated resin (Qiagen). The column was washed with decreasing concentrations of urea. The elution was carried out with imidazole in the absence of urea. The complete

sequence of the proteins of interest may also be cloned into a suitable plasmid and then transferred into the vaccinia virus in order to obtain a recombinant virus.

Use of ligands capable of binding to the proteins of interest identified in the present invention.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for treating mammals suffering from an autoimmune disease, preferably multiple sclerosis, comprising:

(i) either at least one ligand capable of binding to the proteins and/or fragments of the proteins chosen from the target proteins SEQ ID No. 2, 4, 8, 9, 14 and 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, the ligand being capable or not of inhibiting the protein activity,

(ii) or at least one polyclonal or monoclonal antibody capable of binding to at least one protein or one of its fragments chosen from the target proteins SEQ ID No. 2, 4, 8, 9, 14 and 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70%

identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29. This antibody may be neutralizing or not, that is to say capable or not of inhibiting the activity of the protein of interest. The ligand may be chosen from any molecule or molecule fragment capable of binding to the target proteins, for example the receptor for this proteins, the cofactors for these proteins, the polyclonal or monoclonal antibodies capable of binding to the proteins or any fragment of these proteins.

These antibodies are very useful in particular for allowing the use of therapeutic compositions because they lead, for example, to immune reactions directed specifically against immunodominant epitopes or against antigens exhibiting high variability. There are administered to the patient either neutralizing soluble antibodies in order to inhibit their function, or specific soluble antibodies in order to eliminate the peptide by formation of immune complexes. The invention describes the use of antibodies capable of specifically recognizing at least one protein described in the present invention for the treatment and/or for the therapeutic monitoring of a degenerative and/or neurological and/or autoimmune disease, preferably multiple sclerosis. These antibodies are polyclonal and preferably monoclonal. Preferably, these antibodies recognize the active site of the protein and, upon binding, inhibits the function of the protein. The capacity of the antibody to specifically bind to the protein is analyzed by conventional techniques which have been described, such as for example by ELISA or Western blot tests using the natural or synthetic immunogenic peptide or protein. The antibody titer is determined. The capacity of the antibody to neutralize the function of the protein may be analyzed by various means, for example by determining the reduction in the activity of the immunogenic peptide or protein in the

presence of antibodies, preferably by determining the reduction in the gliotoxic activity using the bioassay test *in vitro*.

For example, the monoclonal antibodies directed against the target protein or a portion of this protein are produced by conventional techniques used to produce antibodies against surface antigens. Mice or rabbits are immunized (i) either with the natural or recombinant protein of interest, (ii) or with any immunogenic peptide of this protein of interest, (iii) or with murine cells which express the protein or the peptide of interest and the MHCII molecules.
The Balb/c murine line is the most frequently used. The immunogen is also a peptide chosen from the peptides defined from the primary sequences of the proteins of interest. For example, the following immunogen was prepared: the peptides SEQ ID Nos. 58, 59, 60 derived from the sequence of the GM2 activator protein, the peptides SEQ ID Nos. 61, 62 derived from the sequence of saposin B and the peptides SEQ ID Nos. 63, 64, 65 derived from calgranulin B were coupled to Keyhole Lymphet hemocyanin, abbreviated peptide-KLH, as support for its use in immunization, or coupled to human serum albumin, abbreviated peptide-HSA. The animals were subjected to an injection of peptide-KLH or of peptide-HSA using complete Freund's adjuvant (CFA). The sera and the hybridoma culture supernatants derived from animals immunized with each peptide were analyzed for the presence of anti-protein antibodies by an ELISA test using the initial proteins. The spleen cells of these mice were consequently recovered and fused with myeloma cells. Polyethylene glycol (PEG) is the fusion agent most frequently used. The hybridomas producing the most specific and the most sensitive antibodies are selected. The monoclonal antibodies may be produced *in vitro* by cell culture of the hybridomas produced or by recovering murine ascitic fluid after intraperitoneal injection of the hybridomas in mice. Whatever the mode

of production, in supernatant or in ascites, it is then important to purify the monoclonal antibody. The methods of purification used are essentially ion-exchange gel filtration or exclusion chromatography, or even immunoprecipitation. For each antibody, the method which will make it possible to obtain the best yield should be chosen. A satisfactory number of anti-protein antibodies are targeted in functional tests in order to identify the most efficient antibodies for binding the protein of interest and/or for blocking the activity of the protein of interest. The monoclonal antibodies selected are humanized by standard "CDR grafting" methods (protocol performed by many companies, as a service). These humanized antibodies may be clinically tested in the patient. The efficiency of these antibodies may be monitored by clinical parameters.

The *in vitro* production of antibodies, of antibody fragments or of antibody derivatives, such as chimeric antibodies, produced by genetic engineering, in eukaryotic cells has been described (EP 120 694 or EP 125 023) and is also applicable to the present invention.

Use of molecules inhibiting the proteins of interest identified in the present invention.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for treating mammals suffering from a degenerative and/or neurological and/or autoimmune disease, preferably multiple sclerosis, said composition comprising (i) either at least one molecule inhibiting the function of at least one protein chosen from the proteins identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator

protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, for example inhibiting the gliotoxic activity, (ii) or at least one molecule regulating the expression of at least one protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, for example to block transcription or translation, (iii) or at least one molecule regulating the metabolism of at least one protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, (iv) or at least one molecule regulating the expression and/or the metabolism of a ligand for at least one protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences

belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to SEQ ID No. 8 and SEQ ID No. 10 to 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, for example a receptor or a cofactor. It is also possible to think that these proteins of the human body can be inhibited with no side effect.

Another important aspect of the invention relates to the identification and the evaluation of the therapeutic efficacy of natural and/or synthetic substances (i) capable of blocking and/or inhibiting the activity of the proteins of interest of the invention and/or of their fragment: SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29 and/or (ii) capable of inhibiting their metabolism such as the inhibitors of the corresponding metabolism, the inhibitors of enzymes activated by the coenzymes, (iii) capable of regulating the expression of the proteins of interest (SEQ ID No.

2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, (iv) capable of inhibiting the function and/or the expression of the ligands for the proteins of interest SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, such as for example receptors. These substances may be used in prophylactic or therapeutic treatments of the disease. The invention also relates to methods for treating and preventing an autoimmune disease, for example MS, by administering effective quantities of these substances. The substances may be proteins, antibodies, small synthetic or natural molecules, derivatives of the proteins identified in this invention, lipids, glycolipids and the like. The small molecules may be screened and identified in a large quantity using chemical combinatory libraries. The invention also relates to pharmaceutical compositions comprising these substances in association with acceptable physiological carriers, and methods for the preparation of medicaments to be

used in the therapy or in the prevention of autoimmune diseases including MS using these substances.

To identify inhibitory molecules of low molecular weight such as candidate drugs for degenerative and/or neurological and/or autoimmune diseases, such as multiple sclerosis, there are used the tests and protocols described in above and in the patent applications incorporated by way of reference, using samples collected from untreated or treated patients, untreated or treated animal models, or tissues of untreated or treated animal models. This aspect of the invention also includes a method for identifying substances capable of blocking or inhibiting the activity of the proteins of interest, comprising the introduction of these substances into a test *in vitro* or into an animal model *in vivo*. The molecules selected are tested at different concentrations. These inhibitors are also tested in toxicity and pharmacokinetic assays to know if they can represent valid candidate drugs. The substances tested for the inhibition or the blocking of the protein activities or for the expression of the proteins, in these screening procedures, may be proteins, antibodies, antibody fragments, small synthetic or natural molecules, derivatives of the proteins of interest and the like. The small molecules may be screened and identified in a large quantity using chemical combinatory libraries.

By way of example, there may be mentioned as inhibitory substances:

The inhibitors of the proteins identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24), the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ

ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and the inhibitors of the fragments of said proteins. These inhibitors may be included in a prophylactic and therapeutic composition, in particular for the treatment of multiple sclerosis. For example, lycorine, an alkaloid extracted from Amaryllidaceae (e.g.: Crinum asiaticum) is used *in vitro* at a concentration of between 0.1 and 0.5 µg/ml and *in vivo* at a concentration of between 0.1 and 1 mg/kg/day. For example, Rolipram (trade name) and Ibudilast (trade name), which are two molecules of the same family of inhibitors of 4(PDE4) phosphodiesterases, are used *in vitro* at concentrations of between 1 and 10 µM/l and *in vivo* at concentrations of between 10 mg/kg/day.

From the amino acid sequences of the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29), it is evident that it is possible to deduce the DNA and RNA nucleotide sequences (SEQ ID No. 30, 31, 42, 53) corresponding to the proteins of interest and the sequences encoding the proteins of the family of these proteins of interest (for example SEQ ID No. 32 to 41, SEQ ID No. 43 to 52, SEQ ID No. 54 to 57, SEQ ID No. 66 to 67), taking into account the genetic code and its degeneracy. Thus, the present invention relates to the use of these nucleotide sequences in the form:

- of antisense sequences,

- of sequences encoding a therapeutic gene,

- of sequences which may be contained in a vector for carrying out cell transformation ex vitro and/or in vivo (gene therapy).

Use of nucleic acids deduced from the amino acid sequences of the proteins of interest identified in the present invention; antisense nucleic acids and/or nucleic acids encoding a therapeutic gene.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for treating mammals suffering from a degenerative and/or neurological and/or autoimmune disease, in particular multiple sclerosis, the composition comprising (i) either at least one nucleic acid sequence capable of hybridizing with a nucleic acid sequence encoding the proteins of interest (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, or their fragment(s), (ii) or at least one nucleic acid sequence comprising at least one gene of therapeutic interest encoding the proteins or a fragment of proteins (SEQ ID No. 2, 4, 8, 9, 17, 24), the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16,

SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and elements ensuring the expression of said gene *in vivo* in target cells intended to be genetically modified by said nucleic sequence.

The expression nucleic acid sequence is understood to mean a DNA and/or RNA fragment which is double-stranded or single-stranded, linear or circular, natural and isolated or synthetic, designating a precise succession of nucleotides, modified or otherwise, which makes it possible to define a fragment or a region of a nucleic acid chosen from the group consisting of a cDNA; a genomic DNA; a plasmid DNA; a messenger RNA. These nucleic acid sequences are deduced from the amino acid sequence of the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, using the genetic code. Because of the degeneracy of the genetic code, the invention also encompasses equivalent or homologous sequences. These defined sequences allow persons skilled in the art themselves to define the appropriate nucleic acids.

Accordingly, the present invention relates to a biological material for the preparation of pharmaceutical compositions comprising at least one

nucleic acid sequence capable of hybridizing with a nucleic acid sequence encoding the proteins of interest or their fragment(s) (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29.

The invention consists in defining and using nucleic acid molecules complementary to the DNA and/or RNA sequences encoding the proteins of interest or their fragment(s). These fragments correspond to ribozyme or antisense molecules and may be synthesized using automated synthesizers, such as those marketed by the company Applied Biosystem. The invention describes the use of these nucleic acids capable of hybridizing under stringent conditions with the DNA and/or RNA encoding the proteins of the invention or their fragment(s). Characteristic stringency conditions are those which correspond to a combination of the temperature and of the saline concentration chosen approximately between 12 and 20°C under the Tm ("melting temperature") of the hybrid under study. Such molecules are synthesized and may be labeled using conventional labeling methods used for molecular probes, or may be used as primers in amplification reactions. The sequences which exhibit at least 90% homology relative to a reference sequence also form part of the invention, as well as the fragments of these sequences which have at least 20 nucleotides and preferably 30 contiguous nucleotides that are homologous with respect to a reference sequence. To reduce the proportion of natural or

variant peptides, it is possible to envisage an antisense and/or ribozyme approach. Such an approach is widely described in the literature. Of course, such antisense molecules may constitute, as such, vectors. It is also possible to use vectors which comprise a nucleic acid sequence which encodes an antisense.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for treating mammals suffering from a degenerative and/or neurological and/or autoimmune disease, such as multiple sclerosis, said composition comprising at least one nucleic acid sequence containing at least one gene of therapeutic interest and elements ensuring the expression of said gene *in vivo* in target cells intended to be genetically modified by said nucleic sequence.

These nucleic acid sequences and/or vectors (antisense or encoding a protein or a fragment of a protein) make it possible to target the cells in which the peptide is expressed, such as macrophage cells: (i) either by the use of a targeting molecule introduced on the vector, (ii) or by the use of a particular property of these cells.

Use of vectors comprising a gene of therapeutic interest corresponding to the genes for the proteins of interest identified in the present invention.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for preventing and treating degenerative and/or neurological and/or autoimmune diseases, such as multiple sclerosis, the composition comprising a nucleic acid sequence comprising a gene of therapeutic interest and elements for expressing said gene of interest. The genes may be nonmutated or mutated. They may also consist of nucleic acids modified such that it

is not possible for them to integrate into the genome of the target cell, or of nucleic acids stabilized with the aid of agents, such as spermine.

Such a gene of therapeutic interest encodes in particular:

(i) either at least one protein chosen from the proteins identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, or their fragment(s),

(ii) or at least all or part of a polyclonal or monoclonal antibody capable of binding to at least one protein or a protein fragment chosen from the proteins identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29. This may include in particular a native transmembrane antibody, or a fragment or derivative of such an antibody, as

long as said antibody, antibody fragment or derivative is expressed at the surface of the genetically modified target cell of the mammal and is capable of binding to a polypeptide present at the surface of a cytotoxic effector cell or of a helper T lymphocyte involved in the process for activating such a cell,

(iii) or at least one molecule inhibiting at least one protein or its fragments, said protein being chosen from the proteins identified (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29; the proteins inhibiting the function and/or the metabolism and/or the binding of the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29,

(iv) or at least one ligand or any portion of a ligand capable of binding to at least one protein or one protein fragment chosen from the proteins identified (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide

sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and/or of inhibiting its function.

More particularly, the expression antibody fragment is understood to mean the F(ab)2, Fab', Fab, sFv fragments (Blazar et al., 1997, Journal of Immunology 159: 5821-5833; Bird et al., 1988 Science 242: 423-426) of a native antibody and the expression derivative is understood to mean, for example, a chimeric derivative of such an antibody (see for example the chimeras of the Mouse/Human anti-CD3 antibodies in Arakawa et al., 1996 J Biochem 120: 657-662 or the immunotoxins such as sFv-toxin by Chaudary et al 1989, Nature 339: 394-397). The expression transmembrane antibody is understood to mean an antibody in which at least the functional region capable of recognizing and binding to its specific antigen is expressed at the surface of the target cells in order to allow said recognition and binding. More particularly, the antibodies according to the present invention consist of fusion polypeptides comprising the amino acids defining said functional region and an amino acid sequence (transmembrane polypeptide) allowing anchoring within the membrane lipid double layer of the target cell or at the outer surface of this bilayer. The nucleic sequences encoding numerous transmembrane polypeptides are described in the literature. According to a most advantageous case, the nucleic acid sequence encoding the antibody heavy chain is fused with the nucleic acid sequence encoding the said transmembrane polypeptide.

The expression elements ensuring the expression of said gene *in vivo* refers in particular to the elements necessary to ensure the expression of said gene after its transfer into a target cell. This includes in particular promoter sequences and/or regulatory sequences which are efficient in said cell, and optionally the sequences required to allow expression at the surface of the target cells of said polypeptide. The promoter used may be a viral, ubiquitous or tissue-specific promoter or a synthetic promoter. By way of example, there may be mentioned promoters such as the promoters of the viruses RSV (Rous Sarcoma Virus), MPSV, SV40 (Simian Virus), CMV (Cytomegalovirus) or of the vaccinia virus, the promoters of the gene encoding muscle creatine kinase, actin. It is, in addition, possible to choose a promoter sequence specific for a given cell type, or activable under defined conditions. The literature provides a large amount of information relating to such promoter sequences.

Moreover, said nucleic acid may comprise at least two sequences, which are identical or different, exhibiting a transcriptional promoter activity and/or at least two genes, which are identical or different, situated relative to each other contiguously, apart, in the same direction or in the opposite direction, provided that the transcriptional promoter function or the transcription of said genes is not affected.

Likewise, in this type of nucleic acid construct, it is possible to introduce "neutral" nucleic sequences or introns which do not adversely affect the transcription and are spliced before the translational step. Such sequences and their uses are described in the literature (reference: PCT patent application WO 94/29471).

Said nucleic acid may also comprise sequences required for intracellular transport, for replication and/or for integration, for transcription and/or translation. Such sequences are well known to persons skilled in the art.

Moreover, the nucleic acids which can be used according to the present invention may also be nucleic acids modified such that it is not possible for them to integrate into the genome of the target cell or nucleic acids stabilized with the aid of agents, such as, for example, spermine, which, as such, have no effect on the efficiency of the transfection.

According to one embodiment of the invention, the nucleic acid sequence is a naked RNA or DNA sequence, that is to say which is free of any compound facilitating its introduction into cells (transfer of nucleic acid sequence). However, according to a second embodiment of the invention, to promote its introduction into the target cells and to obtain the genetically modified cells of the invention, this nucleic acid sequence may be in the form of a "vector" and more particularly in the form of a viral vector, such as, for example, an adenoviral vector, a retroviral vector, a vector derived from a poxvirus, in particular derived from the vaccinia virus or from the Modified Virus Ankara (MVA) or from a nonviral vector such as, for example, a vector consisting of at least one said nucleic acid sequence complexed or conjugated with at least one carrier molecular substance selected from the group consisting of a cationic amphiphile, in particular a cationic lipid, a cationic or neutral polymer, a practical polar compound chosen in particular from propylene glycol, polyethylene glycol, glycerol, ethanol, 1-methyl-L-2-pyrrolidone or their derivatives, and an aprotic polar compound chosen in particular from dimethyl sulfoxide (DMSO), diethyl sulfoxide, di-n-propyl sulfoxide, dimethyl sulfone, sulfolane, dimethylformamide, dimethylacetamide,

tetramethylurea, acetonitrile or their derivatives. The literature provides a large number of examples of such viral and nonviral vectors.

Such vectors may in addition and preferably comprise targeting elements which can make it possible to direct the transfer of a nucleic acid sequence toward certain cell types or certain particular tissues such as cytotoxic cells and antigen-presenting cells). They can also make it possible to direct the transfer of an active substance toward certain preferred intracellular compartments such as the nucleus, the mitochondria or the peroxisomes, for example. This may also include elements facilitating penetration into the cell or the lysis of intracellular compartments. Such targeting elements are widely described in the literature. This may include, for example, all or part of lectins, peptides, in particular the peptide JTS-1 (see PCT patent application WO 94/40958), oligonucleotides, lipids, hormones, vitamins, antigens, antibodies, ligands specific to membrane receptors, ligands capable of acting with an antiligand, fusogenic peptides, nuclear localization peptides or a composition of such compounds.

Use of cells transformed *in vivo* after injection of vectors containing at least one gene of therapeutic interest defined from the proteins of interest identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and

advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for preventing and treating mammals suffering from degenerative and/or neurological and/or autoimmune diseases, preferably multiple sclerosis, the composition comprising at least one vector containing a therapeutic gene as described below, capable of being introduced into a target cell *in vivo* and of expressing the gene of therapeutic interest *in vivo*. The advantage of this invention consists in the possibility of maintaining long term a basal level of molecules expressed in the patient treated. Vectors or nucleic acids encoding genes of therapeutic interest are injected. These vectors and nucleic acids should be transported up to the target cells and transfect these cells in which they have to be expressed *in vivo*.

The invention relates to the expression *in vivo* of nucleotide sequences and/or vectors as designated in the preceding paragraph, that is to say sequences corresponding to genes of therapeutic interest encoding in particular:

(i) either at least one protein chosen from the proteins identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of

the peptide sequences SEQ ID No. 1 to 29, or their fragment(s),

(i) or at least all or part of a polyclonal or monoclonal antibody capable of binding to at least one protein chosen from the proteins identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29. This may include a native transmembrane antibody, or a fragment or derivative of such an antibody, as long as said antibody or antibody fragment or derivative is expressed at the surface of the genetically modified target mammalian cell and in that said antibody is capable of binding to a polypeptide present at the surface of a cytotoxic effector cell or of a helper T lymphocyte and involved in the process of activating such a cell. This may include antibody fragments expressed by cells capable of secreting said antibodies in the bloodstream of a mammal or patient carrying the cells genetically modified by the gene encoding the antibody,

(ii) or at least one molecule inhibiting at least one protein chosen from the proteins identified (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No.

10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29; protein inhibiting the function and/or metabolism and/or binding of the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29,

(iii) or at least one ligand or any portion of the ligand capable of binding to at least one protein chosen from the proteins identified (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and/or of inhibiting its function.

According to a particular embodiment, this includes using gene therapy so as to direct the immune response against the target protein, peptide or molecule of interest, that is to say against any protein chosen

from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, their fragment(s) and/or against any molecule inhibiting the function and/or expression and/or metabolism of said proteins of interest, and/or ligands of said proteins such as, for example, the receptors. For that, it is evident that the cells to be targeted for the transformation with a vector are cells belonging to the immune system, either lymphocyte-type cells (CD4/CD8), or antigen-presenting cells (dendritic cells, macrophages and the like).

According to a particular embodiment, the antigen-presenting cells (APC) are genetically modified, in particular *in vivo*. APCs such as macrophages, dendritic cells, microgliocytes and astrocytes play a role in initiating the immune response. They are the first cellular components which capture the antigen, prepare it in the cell and express the transmembrane MHCI and MHCII molecules involved in presenting the immunogen to the CD4+ and CD8+ T cells, they produce specific secondary proteins which participate in activating the T cells (Debrick et al., 1991, J. Immunol 147: 2846; Reis et al., 1993, J Ep Med 178: 509; Kovacsovics-bankowski et al., 1993, PNAS 90: 4942; Kovacsovics-bankowski et al., 1995 Science 267: 243; Svensson et al., 1997, J Immunol 158: 4229; Norbury et al., 1997, Eur J Immunol 27: 280). For a vaccination, it may be advantageous to have a gene therapy system which can target the gene transfer into such APC cells, that is

to say a gene which encodes a polypeptide which can, after its intracellular production and its "processing", be presented to the CD8+ and/or CD4+ cells by the molecules of the MHCI and MHCII complexes, respectively, at the surface of these cells.

It is chosen to express at the surface of the APC cells *in vivo* all or part of an antibody and/or of a ligand such as, for example, a receptor, capable of reacting with the target protein or peptide chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29. Such cells will then specifically phagocytose said protein or said peptide, the "processer" so that fragments of this peptide are present at the surface of the antigen-presenting cells.

The literature provides a large number of examples of genes encoding antibodies capable of reacting with polypeptides or receptors. It is within the capability of persons skilled in the art to obtain the nucleic acid sequences encoding such antibodies. There may be mentioned, for example, the genes encoding the light and heavy chains of the antibody YTH 12.5 (anti-CD3) (Routledge et al. 1991, Eur J Immunol 21: 2717-2725), of the anti-CD3 according to Arakawa et al; 1996, J. Biochem. 120: 657-662. The nucleic acid sequences of such antibodies are easily identifiable from the databases commonly used by persons skilled in the art. It is also possible, starting with hybridomas available

from ATCC, to clone the nucleic acid sequences encoding the heavy and/or light chains of these various antibodies by amplification methods such as RT-PCR with the aid of specific oligonucleotides or techniques using cDNA libraries (Maniatis et al., 1982, Molecular cloning. A laboratory manual CSH Laboratory, Cold Spring Harbor, New York). The sequences thus cloned are then available for their cloning into vectors. According to a preferred case of the invention, the nucleic acid sequence encoding the heavy chain of the antibody is fused by homologous recombination with the nucleic acid sequence encoding a transmembrane polypeptide such as the rabies glycoprotein or gp160 (Polydefkis et al., 1990, J Exp Med 171: 875-887). These molecular biology techniques have been fully described.

It is chosen to express at the surface of the APC cells *in vivo* immunogenic fragments corresponding to at least one proteins chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29. For that, it is possible to choose to cause the vector to express either the full-length polypeptide or, preferably, polypeptides selected to react with specific ligands and/or receptors. The immunogenic peptide encoded by the polynucleotide introduced into the cell of the vertebrate *in vivo* may be produced and/or secreted, made ready and then presented to an antigen-presenting cell (APC) in the context of the molecules of the MHC.

The APCs thus transferred *in vivo* induce an immune response directed against the immunogen expressed *in vivo*. The APCs possess different mechanisms for capturing the antigens: (a) capture of the antigens by membrane receptors such as the receptors for immunoglobulins (Fc) or for the complement which are available at the surface of the granulocytes, monocytes or macrophages allowing efficient delivery of the antigen into the intracellular compartments after phagocytosis mediated by the receptors. (b) entry into the APCs by pinocytosis in fluid phase, involving various mechanisms: micropinocytosis, that is to say the capture of small vesicles (0.1 µm) by the clathrin-coated pits, and macropinocytosis, that is to say the capture of larger vesicles (with a size varying graft 0.5 µm and about 6 µm) (Sallusto et al. 1995, J Exp Med 182: 389-400). While micropinocytosis constitutively exists in all cells, macropinocytosis is limited to cellular types such as, for example, the macrophages, dendritic cells, astrocytes, epithelial cells stimulated by growth factors (Racoosin et al., J Cell Sci 1992, 102: 867-880). In this invention, the expression cells capable of macropinocytosis is understood to mean the cells which can carry out the events described above and the cells which can capture macromolecules preferably between 0.5 µm and about 6 µm in the cytoplasm.

According to a particular embodiment, the cytotoxic effector cells or the helper T lymphocytes are genetically modified in particular *in vivo* so that they express at their surface a polypeptide corresponding to the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to

23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, ligands for said proteins, which are naturally not expressed by these cells and which are capable of inducing the process of activation of such cells, by introducing into these cells nucleic acid sequences containing the gene encoding such a polypeptide. In accordance with the present invention, it is also possible to select a nucleic acid sequence containing a gene of therapeutic interest encoding all or part of an antibody directed against a protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, capable of being expressed at the surface of the target cells of the patient to be treated, said antibody being capable of binding to a polypeptide which is naturally not expressed by these cytotoxic effector cells or helper T lymphocytes.

The expression cytotoxic effector cells is understood to designate the macrophages, astrocytes, cytotoxic T lymphocytes (CTL) and killer (NK) cells as well as their derivatives such as, for example, LAKs (Versteeg 1992 Immunology today 13: 244-247; Brittende et al 1996, Cancer 77: 1226-1243). The expression "helper T lymphocytes" is understood to designate in particular the CD4 cells which allow, after activation, the

secretion of factors for activating the effector cells of the immune response. The polypeptides, and in particular the receptors expressed at the surface of these cells and which are involved in the activation of such cells, constitute in particular all or part of the TCR complex or CD3, all or part of the CD8, CD4, CD28, LFA-1, 4-1BB (Melero et al., 1998, Eur J Immunol 28: 1116-1121), CD47, CD2, CD1, CD9, CD45, CD30 and CD40 complexes, all or part of the cytokine receptors (Finke at al., 1998, Gene therapy 5: 31-39), such as IL-7, IL-4, IL-2, IL-15 or GM-CSF, all or part of the receptor complex for the NK cells such as for example NKAR, Nkp46, and the like; (Kawano et al., 1998 Immunology 95: 5690-5693; Pessino et al., 1998 J Exp Med 188: 953-960), Nkp44, all or part of the macrophage receptors such as for example the Fc receptor (Deo et al., 1997, Immunology Today 18: 127-135).

Numerous tools have been developed for introducing various heterologous genes and/or vectors into cells, in particular mammalian cells. These techniques may be divided into two categories: the first category involves physical techniques such as microinjection, electroporation or particle bombardment. The second category is based on the use of molecular and cell biology techniques with which the gene is transferred with a biological or synthetic vector which facilitates the introduction of the material into the cell in vivo. Nowadays, the most efficient vectors are the viral, in particular adenoviral and retroviral, vectors. These viruses possess natural properties for crossing the plasma membranes, avoiding degradation of their genetic material and introducing their genome into the nucleus of the cell. These viruses have been widely studied and some are already experimentally used in human applications in vaccination, immunotherapy, or to compensate for genetic deficiencies. However, this viral approach has limitations, in particular due to the restricted cloning capacity in these viral genomes,

the risk of disseminating the viral particles produced in the body and the environment, the risk of artefactual mutagenesis by insertion into the host cell in the case of retroviruses, and the possibility of inducing a high inflammatory immune response in vivo during the treatment, which limits the number of injections possible (McCoy et al. 11995, Human Gene Therapy 6: 1553-1560; Yang et al., 1996 Immunity 1: 433-422). Other alternative systems to these viral vectors exist. The use of nonviral methods such as, for example, coprecipitation with calcium phosphate, the use of receptors which mimic the viral systems (for a summary see Cotten and Wagner 1993, Current Opinion in Biotechnology, 4: 705-710), or the use of polymers such as polyamidoamines (Haensler and Szoka 1993, Bioconjugate Chem 4: 372-379). Other nonviral techniques are based on the use of liposomes whose efficiency for the introduction of biological macromolecules such as DNA, RNA, proteins or active pharmaceutical substances has been widely described in the scientific literature. In this domain, teams have proposed the use of cationic lipids having a high affinity for the cell membranes and/or nucleic acids. Indeed, it has been shown that a nucleic acid molecule itself could cross the plasma membrane of some cells *in vivo* (WO 90/11092), the efficiency depending in particular on the polyanionic nature of the nucleic acid. Since 1989 (Felgner et al., Nature 337: 387-388), cationic lipids have been proposed to facilitate the introduction of large anionic molecules, which neutralizes the negative charges on these molecules and promotes their introduction into the cells. Various teams have developed such cationic lipids: DOTMA (Felgner et al., 1987, PNAS 84: 7413-7417), DOGS or Transfectam™ (Behr et al., 1989, PNAS 86: 6982-6986), DMRIE and DORIE (Felgner et al., 1993 methods 5: 67-75), DC-CHOL (Gao and Huang 1991, BBRC 179: 280-285), DOTAP™ (McLachlan et al., 1995, Gene therapy 2: 674-622) or Lipofectamine™, and the other molecules

described in patents WO9116024, WO9514651, WO9405624. Other groups have developed cationic polymers which facilitate the transfer of macromolecules, in particular anionic macromolecules, into cells. Patent WO95/24221 describes the use of dendritic polymers, the document WO96/02655 describes the use of polyethyleneimine or polypropyleneimine and the documents US-A-5595897 and FR 2719316, the use of polylysine conjugates.

Given that it is desired to obtain *in vivo* a transformation targeted toward a given cell type, it is evident that the vector used should be able to be "targeted" itself, as described above.

Use of cells transformed *in vitro* or *ex vivo* with vectors containing a gene of therapeutic interest defined in relation to the proteins of interest identified in the present invention (SEQ ID No. 2, 4, 8, 9, 17, 24) and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29.

The present invention relates to a biological material for the preparation of pharmaceutical compositions for preventing and treating degenerative and/or neurological and/or autoimmune diseases, preferably multiple sclerosis, the composition comprising at least one cell, in particular a cell not naturally producing antibodies, in a form allowing their administration into the body of a mammal, human or animal, as well as

optionally their prior culture, said cell being genetically modified *in vitro* by at least one nucleic acid sequence containing at least one gene encoding *in vivo*:

(i) at least one protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29 and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and any fragment,

(ii) at least one peptide defined from the primary sequence of at least one protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29,

(iii) at least any molecule inhibiting the function and/or binding and/or expression of these proteins,

(iv) at least one peptide derived from the primary sequence of a protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24 and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29, and capable of binding to at least one glycoprotein of the MHCI,

(v) at least any antibody and any portion of antibody which are capable of binding to at least one protein chosen from the proteins SEQ ID No. 2, 4, 8, 9, 17, 24, and the peptide sequences or the fragments of said sequences belonging to the same family of proteins chosen from Perlecan, the precursor of the retinol-binding plasma protein, GM2 activator protein, calgranulin B and saposin B (for example SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5 to 7, SEQ ID No. 10 to 16, SEQ ID No. 18 to 23, SEQ ID No. 25 to 29) and the peptide sequences which exhibit at least 70% identity, preferably at least 80% identity and advantageously at least 98% identity with any one of the peptide sequences SEQ ID No. 1 to 29.

More particularly, said target cell is obtained either from the mammal to be treated, or from a mammal other than that to be treated. In the latter case, it should be noted that said target cell will have undergone a treatment making it compatible with the mammal to be treated. The expression "mammal" is preferably understood to mean a human mammal. These cells are established as cell lines and are preferably MHCII+ or

MHCII+-inducible such as the lymphocytes, monocytes, astrocytes and oligodendrocytes.

The invention also relates to the modified cells and to a method for preparing a cell as described above, characterized in that there is introduced into a mammalian cell not naturally producing antibodies, by any appropriate means, at least one nucleic acid sequence containing at least one gene of therapeutic interest and elements ensuring the expression of said gene in said cell, said gene of therapeutic interest containing a nucleic acid sequence encoding a molecule or a molecule fragment *in vivo*, as described immediately above. More particularly, it relates to prokaryotic cells, yeast cells and animal cells, in particular mammalian cells transformed with at least one nucleotide sequence and/or one vector as described above.

According to a particular embodiment, the cells (dendritic cells, macrophages, astrocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, and the like) of the patient or allogenic cells are placed in contact with a purified preparation of the target polypeptide, the latter being internalized, made ready and presented at the cell surface associated with the MHCI and/or MHCII molecules and thus to induce a specific immune response against the peptide. The "activated" cells are then administered to the patient in whom they will induce an immune response specific for the antigens (a natural route is used for the immune response, but what the antigen-presenting cell is going to present is checked).

According to a particular embodiment, the antigen-presenting cells (dendritic cell, macrophage, astrocytes, and the like) are modified *in vitro* in order to express the antigens in the transformed cell which will associate with the MHCI and/or MHCII

molecules and be presented at the surface of the cells to induce a perfectly targeted immune reaction in the patient in whom the modified cell is administered.

All the vaccine approaches are not always satisfactory and lead, for example, to limited immune reactions directed solely against immunodominant epitopes or against antigens exhibiting great variability. Likewise, the incorrect presentation of the antigens by the glycoproteins of the MHC system at the surface of the cells does not make it possible to develop in the treated patient a suitable anti-protein of interest immunity. To overcome these problems, some authors have proposed, in the context of such vaccine methods, to select the antigenic minimal fragments corresponding to the peptide portions capable of being specifically recognized by the cytotoxic T lymphocytes, expressing them in the cells so that they associate with the molecules of the MHCI and are presented at the surface of the cells in order to induce a perfectly targeted immune reaction in the treated patient(Toes et al. 1997, PNAS 94: 14660-14665). More particularly, it has been shown that epitopes of very small sizes (varying from 7 to about 13 amino acids), which are expressed from minigenes introduced into a vaccinia virus, could induce a cellular type immunization. It has moreover been shown that several minigenes could be conjointly expressed starting with the same vector (this particular construct is called "string of beads"). Such a construct has the advantage of inducing a synergistic CTL-type immune reaction (Whitton et al., 1993 J. of Virology 67: 348-352).

Protocol for bringing the cells and the antigenic fragment into contact:

The presentation of the antigenic fragments by the MHCI molecules depends on an identified intracellular method (see Groettrup et al., 1996 Immunology Today 17: 429-

435 for a review) in which very short antigenic peptides (about 7-13 amino acids) are produced by degradation of a more complex polypeptide against which the final immune reaction will be directed. These short peptides are then combined with the MHCI or MHCII molecules to form a protein complex which is transported to the cell surface in order to present said peptides to the circulating cytotoxic T lymphocytes or to the circulating helper T lymphocytes, respectively. It should be noted, in addition, that the specificity of the MHCI or MHCII molecules toward the antigenic peptides varies as a function of the MHCI or MHCII molecules (example for MHCI: HLA-A, HLA-B, and the like) and the allele (example for MHCI: HLA-A2, HLA-A3, HLA-A11) which are considered. Within the same animal species, from one individual to another, there is great variability of the genes encoding the molecules of the MHC system (on this subject, see in particular George et al., 1995, Immunology Today 16: 209-212).

According to a particular embodiment, the cells, such as dendritic cells, macrophages, astrocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, are modified so as to express at their surface antibodies specific for the targeted peptide. The peptide is neutralized with the antibodies expressed at the surface of the cells. These cells are preferably immune cells, preferably from the patient, are preferably cytotoxic and modified to express all or part of an antibody specific for the target polypeptide.

Isolation of mononucleated cells from peripheral blood:

In 1968, Boyum described a rapid technique which makes it possible, by centrifugation of blood on a density gradient, to separate the mononucleated cells (lymphocytes and monocytes) with a good yield (theoretical yield 50%, that is to say 10^6 cells/ml of

blood). 50 ml of peripheral blood sterilely collected in heparinized tubes are centrifuged for 20 minutes at 150 g at 20°C. The cells recovered are diluted in two volumes of initial peripheral blood of sterile PBS. 10 ml of this suspension are deposited on 3 ml of a Ficoll-Hypaque solution (medium for separation of the lymphocytes, Flow). After centrifuging for 20 minutes at 400 g and 20°C without decelerating braking, the mononucleated cells sediment at the PBS-Ficoll interface, as an opalescent dense layer, whereas practically all the red blood cells and the polynuclear cells sediment at the bottom of the tube. The mononucleated cells are recovered and washed with sterile PBS.

Internalization of the antigens by the antigen-presenting cells:

Prior treatment of the antigen-presenting cells: the antigen-presenting cells are washed beforehand with PBS buffer containing 0.5% (w/v) BSA, then counted and they are then preincubated in the presence of various reduction inhibitors three times in PBS-0.5% BSA containing 10 µM to 10 mM final of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) or NEM (N-ethylmaleimide). The subsequent stages of binding of antigens to the cell surface or of internalization of antigens are also carried out in the presence of various concentrations of inhibitors.

Protocol for internalization of the antigens by the antigen-presenting cells:

8×10^6 cells are internalized in the presence of saturating quantity of proteins radiolabeled with iodine 125 (1 µg) in microwells in 70 µl. After incubating for one hour at 4°C, with stirring, the antigens are bound to the surface of the cells. The cell suspension is washed twice in PBS-BSA and the

cellular pellets are taken up in 70 µl of buffer and incubated at 37°C for various periods ranging up to 2 hours. Cells and supernatants are separated by centrifugation at 800 g for 5 minutes 4°C. For longer incubation periods, the preliminary stage of prebinding of the antigens to the surface of the cells is eliminated. The cells are diluted in RPMI-10% FCS medium in the presence of 20 mM Hepes, at 10^6 cells/ml. The cells are incubated in the presence of an excess of antigen for various periods at 37°C (1 µg of molecules/ 5×10^7 monocyte/macrophage cells or 10^8 B-EBV cells).

All the therapeutic agents defined in the context of the present invention are used for preventing and/or treating a degenerative and/or neurological and/or autoimmune disease, such as multiple sclerosis, alone or in combination. They may also be used to evaluate their efficacy *in vitro* or *in vivo*.

Administration of therapeutic agents in humans:

The biological material according to the invention may be administered *in vivo* in particular in injectable form. It is also possible to envisage injection by the epidermal, intravenous, intraarterial, intramuscular or intracerebral route with a syringe or any other equivalent means. According to another embodiment, by oral administration or any other means perfectly known to a person skilled in the art and applicable to the present invention. The administration may take place as a single dose or as a dose repeated once or several times after a certain time interval. The most appropriate route of administration and dosage vary as a function of various parameters such as, for example, the individual or the disease to be treated, the stage and/or the progression of the disease, or alternatively the nucleic acid and/or protein and/or peptide and/or molecule and/or cell to be transferred or the target organ/tissue.

To carry out the treatment of the mammal mentioned in the present invention, it is possible to have pharmaceutical compositions comprising a biological material as described above, advantageously combined with a pharmaceutically acceptable vehicle for administration to humans or to animals. The use of such carriers is described in the literature (see, for example, Remington's Pharmaceutical Sciences 16th ed. 1980, Mack Publishing Co). This pharmaceutically acceptable vehicle is preferably isotonic, hypotonic or exhibits low hypertonicity and has a relatively low ionic strength, such as for example a sucrose solution. Moreover, said composition may contain solvents, aqueous or partially aqueous vehicles such as sterile water, free of pyrogenic agents and dispersion media for example. The pH of these pharmaceutical compositions is suitably adjusted and buffered according to conventional techniques.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents the amino acid sequence of the GM2AP protein (SEQ ID NO: 73), and the localization of the peptides, which is underlined, and which are used for the production of anti-GM2AP peptides antibodies.

Figure 2 represents the amino acid sequence of the MRP14 protein (SEQ ID NO: 75), and the localization of the peptides, which is underlined, and which are used for the production of anti-MRP14 peptides antibodies.

Figure 3 represents the amino acid sequence of the Saposin B protein (SEQ ID NO: 74), and the localization of the peptides, which is underlined, and which are used for the production of anti-Saposin B peptides antibodies.

Figure 4 represents the assay of the MRP8 protein (ng/ml - on the y-axis) in the urine of patients suffering from multiple sclerosis (MS), in the urine of patients suffering from other neurological diseases (OND) and in the urine of controls considered healthy (HC). n means the number of urine samples tested per category.

Figure 5 represents the assay of the MRP14 protein (ng/ml - on the y-axis) in the urine of patients suffering from multiple sclerosis (MS), in the urine of patients suffering from other neurological diseases (OND) and in the urine of controls considered healthy (HC). n means the number of urine samples tested per category.

Figure 6 represents the assay of the MRP8/14 protein (ng/ml - on the y-axis) in the urine of patients suffering from multiple sclerosis (MS), in the urine of patients suffering from other neurological diseases (OND) and in the urine of controls considered healthy (HC). n means the number of urine samples tested per category.

Figure 7 represents the mean concentrations of the MRP8, MRP14 and MRP8/14 proteins (ng/ml - on the y-axis) in the urine of patients suffering from multiple sclerosis (MS), in the urine of patients suffering from other neurological diseases (OND) and in the urine of controls considered healthy (HC). n means the number of urine samples tested per category.

Figure 8 represents the assay of the GM2AP protein (ng/ml - on the y-axis) in the urine of patients suffering from multiple sclerosis (MS), in the urine of patients suffering from other neurological diseases (OND) and in the urine of controls considered healthy (HC). n means the number of urine samples tested per category. MS means multiple sclerosis, OND means other

neurological diseases and Healthy means samples from controls supposed healthy (HC).

Figure 9 represents the assay of the Saposin B protein (µg/ml - on the y-axis) in the urine of patients suffering from multiple sclerosis (MS), in the urine of patients suffering from other neurological diseases (OND) and in the urine of controls considered healthy (HC). n means the number of urine samples tested per category. MS means multiple sclerosis, OND means other neurological diseases and Healthy means samples from controls supposed healthy (HC).

Figure 10 represents the codetection of the Saposin B (µg/ml - on the y-axis) and GM2AP (ng/ml - on the x-axis) proteins in urine samples from MS patients, controls supposed healthy and patients suffering from other neurological diseases and the correlation observed between the levels of the two proteins.

Figure 11 represents: figure 11A, the assay of the GM2AP protein in ng/ml in the urine of an MS patient in progressive remittent form (light-colored curve) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (dark-colored curve); figure 11B, the assay of the Saposin B protein in µg/ml in the urine of an MS patient in progressive remittent form (light-colored curve) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (dark-colored curve).

Figure 12 represents the product of the concentrations of the GM2AP and saposin B proteins in ng×µg/ml^2 in the urine of an MS patient in progressive remittent form (light-colored curve) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (dark-colored curve).

Figure 13 represents: figure 13A, the assay of the GM2AP protein in ng/ml in the urine of an MS patient in progressive remittent form (light-colored curve) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (dark-colored curve); figure 13B, the assay of the Saposin B protein in µg/ml in the urine of an MS patient in progressive form (light-colored curve) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (dark-colored curve).

Figure 14 represents the product of the concentrations of the GM2AP and saposin B proteins in ng×µg/ml^2 in the urine of an MS patient in progressive form (light-colored curve) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (dark-colored curve).

Figure 15 represents the correlation between the concentrations of GM2AP in ng/ml (x-axis) and gliotoxicity as a percentage of dead cells estimated by the MTT test (y-axis) determined in the urine of MS patients and of controls.

Figure 16 represents the correlation between the concentrations of Saposin B in µg/ml (x-axis) and gliotoxicity as a percentage of dead cells estimated by the MTT test (y-axis) determined in the urine of MS patients and of controls.

Figure 17 represents the correlation between the product of the concentrations of GM2AP and Saposin B in ng×µg/ml^2 (x-axis) and gliotoxicity as a percentage of dead cells estimated by the MTT test (y-axis) determined in the urine of MS patients and of controls.

Figure 18 represents the correlation between the concentrations of GM2AP (ng/ml - on the left-hand y-axis), the concentrations of Saposin B (µg/ml -

right-hand y-axis) and the gliotoxicity as a percentage of dead cells estimated by the MTT test (x-axis). Two estimated correlation straight lines are represented on the graph. The lines in bold relate to the concentrations of saposin B; the lines in light black relate to the concentrations of GM2AP.

DETAILED DESCRIPTION OF EMBODIMENTS

Examples:

Example 1: Collecting and pooling of urines

Urine samples of different volumes were collected from healthy individuals (MS-negative) having a priori no neurological or autoimmune disease. The toxic activity of each sample toward murine astrocyte cells was tested *in vitro* using the MTT test. In total, a pool of 20 liters of urine was formed (MS-negative pool). In parallel, urine samples of different volumes were collected from individuals suffering from multiple sclerosis (MS-positive) at various stages of the disease. The toxic activity of each sample toward murine astrocyte cells was tested *in vitro* using the MTT test. In total, a pool of 80 liters of urine was formed (MS-positive pool).

Example 2: Purification of the urinary proteins

The pools of MS-positive and MS-negative urine, collected and tested according to example 1, were purified in order to obtain a high protein concentration and to remove the high molecular weight proteins as far as possible.

Precipitation: precipitations with ammonium sulfate (Prolabo - ref. 21 333 365) were carried out on the pools of MS-positive and MS-negative urine. The percentage of 60% saturated ammonium sulfate per 40% of

urine, that is 390 grams of ammonium sulfate per liter of urine, was used. Each pool was distributed into fractions of 1.8 liters in 2-liter bottles in order to improve the precipitation. The precipitation was carried out for 2 × 8 hours, at room temperature, with gentle stirring. After centrifugation of the pools of urine at 3 000 rpm for 10 min, at a temperature of 10°C, the pellet obtained is taken up in 20 mM Tris buffer containing 1 mM $CaCl_2$ and 0.25 M urea. The mixture was then centrifuged at 3 000 rpm for 10 min. The supernatant contains the concentrated proteins. It is either used immediately for the next stage, or frozen if the next stage cannot be performed continuously.

Ion-exchange chromatography: the solution containing the proteins was then passed over a DEAE fast Flow gel (marketed by PHARMACIA). This stage is carried out at low pressure on a PHARMACIA column filled with gel. The buffers are brought to the column by a peristaltic pump which allows a uniform flow rate. The buffer for equilibrating the column is 20 mM Tris buffer, pH 7. The fraction corresponding to the precipitation supernatant and containing an excessively high quantity of salts is dialyzed against this buffer before depositing on the column. Elution with a salt gradient makes it possible to recover the proteins. The elution gradient is performed in steps of 100, 200, 300, 500 mM NaCl in the buffer for equilibrating the column. The elution fractions are tested by the MTT test and only the positive fractions, that is the fraction eluted at 200 Mm NaCl, will be preserved. These fractions may be immediately treated or stored in the freeze-dried state.

Purification: steric exclusion chromatography based on the difference in size and shape of the proteins to be eluted was used. The fraction corresponding to the 200 mM NaCl elution is deposited on the column. During

the elution, the proteins of low molecular mass are retained and therefore eluted later than the large molecules. The purifications were carried out on HPLC with a TosoHaas TSK Prep G 3000 SW column having a diameter of 21.5 mm and a length of 300 mm, the molecular mass exclusion limit is 500 000 daltons. The elution buffer used contains 100 mM phosphate, 100 mM sodium sulfate, at pH 6.8. The separation of the protein mixture was carried out in 60 min. Only the fraction corresponding to a mass of 15-20 000 daltons was preserved. This fraction is dialyzed in 20 mM Tris buffer containing 0.2 mM $CaCl_2$, pH 7.2, and then freeze-dried.

At each stage, only the fractions having a significant toxic activity were retained for the next stage. The toxic activity of the proteins was checked at each stage using the MTT test. Only the fractions having a significant toxic activity were retained for the additional purification stage described in example 3.

Example 3: Additional purification of the urinary proteins by reverse phase chromatography

Pools of urine from MS patients (MS-positive pool) and from non-MS patients (MS-negative pool), obtained after purification according to example 2, were taken up in distilled water and then diluted with a 0.2% TFA/10% acetonitrile solution in order to obtain a final concentration of about 130 to 140 µg/ml.

The separation by C8 reverse phase HPLC was carried out on a Brownlee Aquapore column (trade name) marketed by the company Perkin Elmer (column characteristics: 300 angstroms/7 µm/(100×4.6) mm). Two separate columns were used for the positive and negative pools respectively. The injections were carried out by multiple injections of 250 µl. The proteins were eluted with a linear gradient from 5% to 15% of buffer B over

5 min, and then from 15% to 100% of buffer B over 95 min, at a flow rate of 0.5 ml/min. The separation buffers A and B used are the buffer 0.1% TFA (Pierce No. 28904)/MilliQ water and the buffer 0.09% TFA/80% acetonitrile (Baker) respectively. The detection was carried out by measuring the UV absorbence at 205 and 280 nm. Fractions were collected in 1.5 ml and 0.5-1 ml fractions in the zone of interest. The fractions were frozen after collection in dry ice.

The fractions collected were then dried in a speed vac and taken up in 100 µl of 0.1% TFA/30% acetonitrile, 20 µl of the fractions were transferred into 500 µl eppendorfs, dried and washed twice with 100 µl of MilliQ water and then dried again.

The toxic activity of the proteins contained in each fraction collected after elution was determined with the aid of the MTT test. Only fraction 21 exhibiting a significant toxic activity was retained. The number for this fraction corresponds to the order of elution as a function of the elution conditions stated in this example.

Example 4: Analysis of the proteins obtained by HPLC separation on SDS-TRICINE gel

The collection pool for fraction 21 obtained by HPLC, as described in example 3, and resulting from 20 injections of the MS-positive pool, was deposited on a precast 16% SDS-TRICINE gel of 10 wells and 1 mm thick (marketed by the company Novex). The conditions for using the gel correspond to those recommended by the supplier. The sample is taken up in 75 µl of 1 times concentrated sample buffer (SDS-TRICINE No. LC 1676, 1 ml two times concentrated + 50 µl of β-mercapto-ethanol (Pierce) diluted 1/2 in water) and 25 µl of the sample are deposited on the gel in three portions. The collection pool for fraction 21 obtained from 6

injections of the MS-negative pool was deposited on the gel under the same conditions as described for the MS-positive pool. The migration on the two gels was carried out in parallel in the same migration tank (XCELL II NOVEX (trade name)) at a constant voltage of 125 mV for 2 hours. The tank is placed in a container containing ice. The gels were stained directly after migration by zinc/imidazole staining (staining kit 161-0440 marketed by the company BIORAD) so as to obtain a reversible negative staining. The protein bands are translucent on an opaque base.

Example 5: Digestion of the gel bands with trypsin

All the protein bands visualized in the deposits of fraction 21 were cut out and subjected to proteolysis with trypsin.

The gel bands are cut out with a scalpel into slices of 1 mm and transferred into eppendorf tubes. The eppendorfs are subjected to a centrifugation peak so as to cause the gel pieces to fall and, after centrifugation, 100 µl of washing buffer (100 Mm NH_4CO_3/50% CH_3CN) are added to the gel pieces. After stirring for 30 min at room temperature, the supernatant is removed in fractions of 20 µl and the washing step is repeated twice. The eppendorfs are dried for 5 min in speed vac. 20 µg of trypsin (modified sequenal grade PROMEGA V5111) are taken up in 200 µl of digestion buffer (5 mM TRIS, pH 8) and are dissolved for 30 min at room temperature, with intermittent stirring, and 20 to 30 µl of resuspended trypsin are added to the gel pieces. The eppendorfs are centrifuged and stored in a hot room at 28°C overnight. After digestion, the gel bands may be used immediately for the measurements of mass or frozen for subsequent use.

Example 6: Chemical digestion of the gel bands with CNBR

In the event of a protein being resistant to enzymatic cleavages, in particular to the action of trypsin as described in example 5, the bands between 16 kD and 20 kD were treated with CNBR. The gel bands, already used for the digestions with trypsin, are dried for 5 to 10 min in speed vac.

A solution of CNBR (FLUKA) at 200 mg/ml was prepared in 70% formic acid (BAKER). 20 µl of this solution were used to rehydrate the gel pieces. The reaction was carried out for 20 h at room temperature and in the dark. The peptides are extracted for 3 times 30 min with 100 µl of 0.1% TFA/60% acetonitrile. The extraction solutions are combined and concentrated to 20 µl. These samples are diluted 5-fold in 0.1% TFA/ water. The separation conditions are those described for the peptides from the digestion with trypsin.

Example 7: Analysis by MALDI-TOF spectrometry

30 µl of extraction buffer (2% TFA/50% acetonitrile) are added to the samples. The eppendorfs to be analyzed are subjected to a centrifugation of 5 min, and then to a sonication of 5 min, and finally to a centrifugation of 1 min.

On a stainless steel disk, 14 deposits of 0.5 µl of matrix (α-cyano-4-hydroxytranscinnamic acid at saturation in acetone) are carried out. A fine uniform microcrystalline layer is obtained. 0.5 µl of a solution of 2% TFA/water are deposited on this sublayer on the 14 deposits, and then 0.5 µl of sample to be analyzed are added. 0.5 µl of a solution at saturation with α-cyano-4-hydroxytranscinnamic acid acid in 50% acetonitrile/water is added to this drop thus formed. After drying at room temperature for 30 min, the crystalline deposits are washed with 2 µl of water which are immediately evacuated by a puff of air. All

the spectra are obtained on a BRUKER BIFLEX (trade mark) mass spectrometer equipped with a reflectron. The measurements (90 to 120 laser shots on the entire deposit) are accumulated in order to obtain a mass spectrum which is most representative of all the peptides present in the matrix-sample sandwich. For each deposit, a calibration with the peptides from the autolysis of trypsin was made in order to be able to use a measurement accuracy of less than 100 ppm.

Searches in databanks were carried out in MS-FIT PROTEINPROSPECTOR (http://prospector.ucsf.edu). The common parameters used in these searches are (1) database: NCBInr, (2) a tolerance of 100-50 ppm, (3) the cysteins are not modified, (4) the methionines may be oxidized, (5) molecular weight range: 1 000-100 000 Da, (6) up to 3 cleavage sites may be ignored.

Example 8: N-terminal sequencing of the digestion peptides

(i) Extraction and separation by HPLC of the digestion peptides.

After the measurements of mass on the entire digestion, the rest of the peptides are extracted 3 times 30 min in a sonication bath with 0.1% TFA/60% acetonitrile. The extraction solutions are combined and dried up to 20 µl in speed vac. After dilution in 80 µl of buffer A (0.1% TFA/water), the extractions of the gel bands, digested with trypsin, are injected onto a C18/MZ-Vydac/(125x1.6) mm/5 µm column. The elution of the peptides is carried out at a flow rate of 150 µl/min, in a gradient ranging from 5% of buffer B (0.09% TFA/80% acetonitrile) to 40% of buffer B over 40 min, and then from 40% of buffer B to 100% of buffer B over 10 min. The detection is made by measuring the UV absorbence at 205 nm. The collection of the peaks is carried out in 500 µl eppendorf tubes. The fractions are stored on ice and, for the band of 18-20 kD of the

MS-positive pool 21, analyzed by MALDI-TOF mass spectrometry.

(ii) N-terminal sequencing

The fractions corresponding only to a single mass peak were analyzed by Edman degradation on a sequencer (model 477A PERKIN ELMER/Applied Biosystems). The sequencing conditions are those described by the manufacturer. A microcartridge was used for depositing the samples and the PTH-amino acids are identified with an online HPLC system (model 120A PERKIN ELMER/Applied Biosystems).

The deposition of the fraction to be sequenced is made in several depositions of 15 µl with intermediate dryings. The tube which contained the peptide is washed with 15 µl of 85% formic acid (BAKER). The amino acid sequences still correspond to the masses measured. The peptides, whose masses do not correspond to the principal protein identified, were sequenced as a priority. In this manner, it was possible to identify up to three proteins in a gel band.

Example 9: Results and discussion.

After reversed HPLC of the MS-negative control pool and of the MS-positive pool, the toxic activity of each elution fraction was determined using the MTT test. Only fraction 21 of the MS-positive pool exhibits a toxic activity *in vitro*. Fraction 21 of the MS-negative control pool exhibits no toxic activity. The toxic activity of fraction 21 of the MS-positive pool was confirmed *in vitro* by FACS, as described in patent application WO 98/11439 on murine astrocyte cells.

The protein content of fraction 21 of the MS-negative control pool and of the MS-positive pool was observed after separation on 16% SDS-TRICINE gel and staining of

the gel with zinc/imidazole. Proteins of high apparent molecular weights were found in the two fractions. On the other hand, five different bands of low apparent molecular weights are only visible in fraction 21 of the MS-positive pool (bands 8, 14, 18 and 20 kD). To each band there corresponds at least one protein and variants of said proteins which have an apparent molecular weight close to that of the native protein. These variant sequences exhibit a percentage homology or identity with the native sequences of at least 70%, preferably of at least 80% and advantageously of at least 98%.

The proteins of interest of fraction 21 of the MS-positive pool were then analyzed by mass spectrometry and/or sequencing and searching for homology in the databanks. The results show the presence of five protein bands migrating between 22 and 5 kD in fraction 21 of the MS-positive pool and variants of said proteins.

These proteins are the C-terminal fragment of Perlecan, which starts at amino acid 3464 and ends at amino acid 3707 of the complete protein sequence, identified in the sequence identifier SEQ ID No. 2, the precursor of the retinol-binding plasma protein whose sequence is given in SEQ ID No. 4, the GM2 activator protein identified in SEQ ID No. 8, calgranulin B identified in SEQ ID No. 17 and saposin B represented in SEQ ID No. 24. As described above, homologs or variants of said proteins were also identified by sequencing. These homologous or variant protein sequences are the product of mutations in the genes encoding said proteins. By way of example, SEQ ID No. 9 exhibits 99% homology or identity with SEQ ID No. 8 of the GM2 activator protein and the fragment of SEQ ID No. 9 which starts at amino acid 34 and ends at amino acid 202 exhibits 98.88% homology or identity with the fragment corresponding to the native protein identified in SEQ ID No. 8.

Example 10: Identification of the proteins in a urine sample

Urine samples from an MS-negative individual and from an MS-positive patient were collected. These urine samples were purified according to the protocol described above. The final elution fractions 21 were analyzed separately by mass spectrometry. The mass profile of each fraction corresponding to each urine sample was compared with the mass profile obtained for the proteins identified in the preceding examples. The results show that for the urine sample from the MS-positive patient, the masses correspond to the molecules (i) C-terminal fragment of Perlecan, (ii) GM2 activator protein, (iii) calgranulin B and (iv) saposin B identified above. On the other hand, none of these masses was identified in the mass profile obtained after analysis of the urine sample obtained from the MS-negative individual. The method described can be used as a diagnostic assay.

Example 11: Western blot assay

Western blottings were carried out on different fractions of crude or purified urine as described in example 2. Urine samples from healthy individuals and from patients suffering from multiple sclerosis are tested in parallel. The samples are deposited on an electrophoresis gel which makes it possible to separate the various proteins according to their molecular mass under the action of an electric field. The Western blottings are carried out after transferring the proteins from the gel onto a membrane. To visualize the transferred proteins, the membrane is saturated with saturation buffer and then incubated with an antibody directly labeled with alkaline phosphatase. The antibody used is an anticalgranulin antibody (mouse monoclonal antibody, clone CF 145 subtype IgG 2b

marketed by the company Valbiotech: reference MAS 696p batch PC96G696). The substrate for the enzyme is 3,3'-(1,1'-biphenyl)-4,4'-diazonium dichloride and sodium 2-naphthalenylphosphate (marketed under the name β Naphthyl acid phosphate Sigma ref. N7375 and Tetrazotized ô-dianisine D3502) is added for revealing the bands and visualizing the proteins linked to the antibody. A molecule with an apparent molecular mass of about 14 000 is revealed in the purified urines from patients suffering from MS, with a relatively intense signal. This protein corresponds to calgranulin B (apparent molecular mass: 14 kD). By contrast, no signal is observed from urine from healthy individuals. This observation confirms the presence of this protein specifically in the urines from patients suffering from MS and the use of a method of detection using an antibody recognizing the protein.

Example 12: Production of monoclonal antibodies

The production of monoclonal antibodies using ascites requires compatibility of the H-2 system between the hybridoma and the producing mouse. Twenty 6-week-old female Balb/c mice receive an injection of 0.5 ml of Pristane (2,6,10,14-tetramethylpentadecane acid) in their peritoneal cavity, for the production of ascites (Porter et al., 1972). One week to 10 days later, 5×10^6 to 10×10^6 hybridomas, diluted in 0.5 ml of sterile buffer containing 0.145 M NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_4 at pH 7.4, are injected by the intraperitoneal route. The ascites appear one to two weeks later. The ascitic fluids present in the peritoneal cavity are then collected with a syringe after incision of the peritoneum. The fluid collected is centrifuged at 3 000 g for 15 minutes at room temperature, filtered on gauze in order to remove the fat, and then buffered by adding 1/20th of its volume of 1M Tris-HCl at pH 8.0. This method makes it possible

to obtain quantities of antibody 10 times higher than those obtained by hybridoma culture.

The immunoglobulins present in the ascitic fluid are released by the salts (ammonium sulfate or sodium sulfate). The ascitic fluid is precipitated with 40% ammonium sulfate. After 20 minutes in the cold, the solution is centrifuged for 15 minutes at 8 000 g at 4°C. The precipitate is washed and resuspended in the cold in a 40% ammonium sulfate solution and then centrifuged again. The new precipitate enriched with IgG is redissolved in PBS buffer and dialyzed overnight against the 25 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.4. In parallel, an agarose-Protein A (or protein G) column (marketed in the freeze-dried form, Pierce) is extensively washed with the 25 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.4. The solution enriched with IgG is deposited on the column and then the column is washed. The IgGs retained by the column are eluted at acidic pH (200 mM glycine, pH 2.8). The eluted fractions are neutralized with one volume of 1M Tris-Base, pH 10.5. The immunoglobulin content of each fraction collected is quantified by reading the absorbance at 280 nm (e 1%, 1 cm = 14.0, Prahl and Porter 1968). The rich fractions are pooled. The degree of purification of the pooled IgGs is analyzed by acrylamide gel electrophoresis in the presence of SDS. The purified IgGs are dialyzed overnight against the 25 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.4, sterilely filtered, aliquoted and stored at -20°C. Their final concentration is determined by reading the absorbance at 280 nm or by micro-BCA assay. The immunogenic peptides designated by the references SEQ ID No. 58, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, SEQ ID No. 59, and SEQ ID No. 65 were used for the production of monoclonal antibodies, according to the protocol described above. However, it is in the capability of persons skilled in the art to define other protocols for the production of monoclonal antibodies, for

example using the techniques described by Köhler and Milstein and by Galfre G. *et al.* previously cited or techniques derived therefrom.

Production of recombinant proteins and of polyclonal and monoclonal antibodies

Recombinant proteins:

The recombinant proteins GM2AP (SEQ ID NO. 73) and Saposin B (SEQ ID NO. 74) used to produce the calibration series for this study were produced in a prokaryotic system and purified from the clones of these two proteins obtained in our laboratory using the methods and protocols well known to persons skilled in the art.

Anti-GM2AP or anti-Saposin B antibodies:

The anti-GM2AP or anti-Saposin B antibodies used to carry out the study were produced in our laboratory or generously given.

Anti-Saposin B and anti-GM2AP polyclonal antibodies (Li et al., Glycoconjugate, 1984) were used for the study (cf the examples below): they are called SAP84 and GM2AP84.

Anti-GM2AP or anti-Saposin B polyclonal antibodies were produced and purified in the laboratory using the protocols and methods well known to persons skilled in the art: 50 µg of prokaryotic GM2AP or Saposin B protein purchased were injected into rabbits on days D0, D28 and D56; two booster injections were carried out once per month for two consecutive months. The two anti-GM2AP polyclonal antibodies and two anti-Saposin B polyclonal antibodies were thus obtained and their specificity toward the recombinant protein was verified by Western blotting and Elisa.

Anti-GM2AP or Saposin B peptides polyclonal antibodies were produced and purified in the laboratory using the protocols and methods well known to persons skilled in the art: 75 µg of GM2AP or Saposin B peptides defined, produced and coupled to KLH in our laboratory were injected on days D0, D28 and D56; several boosts were thus carried out once per month for 5 consecutive months with injection of 75 µg each time. Four anti-GM2AP peptides polyclonal antibodies, four anti-Saposin B peptides polyclonal antibodies and four anti-MRP14 peptides rabbit polyclonal antibodies were obtained and their specificity toward the recombinant protein was verified by Western blotting and by Elisa. The sequence of the GM2AP, Saposin B and MRP14 peptides chosen are described in figures 1 to 3.

The following were obtained:

- an antibody anti-mixture of two peptides of 13 and 15 amino acids of GM2AP: 189-190; an antibody anti-peptide of 18 amino acids of GM2AP: 191-192 (cf. figure 1),

- an antibody anti-mixture of two peptides of 13 and 19 amino acids of MRP14: 193; an antibody anti-peptide of 17 amino acids of MRP14: 195-196 (cf. figure 2),

- an antibody anti-mixture of three peptides of 12, 15 and 15 amino acids of Saposin B: 74-75; another antibody anti-mixture of 3 peptides of 12, 15 and 15 amino acids of Saposin B: 72-73 (cf. figure 3).

Anti-native fraction monoclonal antibodies were produced and purified in the laboratory using the protocols and methods well known to persons skilled in the art. The "native fraction" corresponds to the cytotoxic elution fraction obtained from the pool of 80 liters of urine from MS patients and after

purification. It is the last elution fraction which contains the three proteins GM2AP, Saposin B, MRP14. 30 µg of this purification fraction were injected into three mice on days D0, D14, D28 and the sample collection was carried out on D38. After "screening" and cell fusion, protocols known to persons skilled in the art for establishing hybridomas and monoclonal antibodies, the hybridomas were reinjected into the mice and the ascitic fluid was recovered 10 days later. The antibodies were purified on sepharose-Protein A column and the specificity toward the fraction used for the immunization was verified by Western blotting and by Elisa. Thus, four monoclonal antibodies were obtained: 191C1A7, 3D3F9, 18C8C5 and 7D12A8.

Example 13: Assay of the MRP14 proteins in the urines by the ELISA technique

The MRP14, MRP8 proteins and the MRP8/14 heterocomplex were assayed in human urines using (i) either an Elisa assay technique according to the method known to persons skilled in the art and using the anti-MRP antibodies described in the preceding examples; (ii) or the "MRP Enzyme Immunoassay" kit marketed by BMA Biomedicals AG, Augst, Switzerland, using the antibodies of the kit, the protocol being carried out according to the leaflet in the kit.

Detection of MRP14 and MRP8/14 in urines

The assay was carried out using 17 urines of individuals from the active population (HC), 27 urines of patients suffering from multiple sclerosis (MS) and 7 urines of patients suffering from other neurological diseases (OND).

- Figure 4 illustrates the levels of MRP8 assayed in these urines: while the MRP8 concentration is practically zero in the OND urines, there is no real

difference in distribution between the HC and MS urines. It should be noted, however, that the differences observed are practically negligible because the concentrations assayed are extremely low.

- Figure 5 illustrates the levels of MRP14 assayed in the same urines: while there are no real differences in the distribution of the concentrations between the HC and OND urines, the concentrations are higher in certain MS urines.

- Figure 6 illustrates the levels of MRP8/14 heterodimer assayed in the same urines: while there is no real difference between the concentrations of the HC and OND urines, higher concentrations are observed in certain MS urines, perhaps corresponding to a subpopulation of MS patients characterized by an activity of the disease. MRRP8/14 assayed in the urines is a marker for the activity of the MS disease characterized by an inflammation peak).

- The recapitulative figure 7 confirms that there is no significant difference in MRP8 and MRP14 concentration between the HC, OND and MS urines, while a small difference in MRP8/14 concentration is observed between these urines, this concentration being higher on average in the MS urines and being a marker for the activity of the disease (inflammation peak).

Example 14: ELISA protocols used for the assay of the GM2AP and Saposin B proteins

The GM2AP or Saposin B proteins were assayed in human urines using anti-GM2AP or anti-Saposin B? polyclonal antibodies according to the Elisa protocol described by Gardas et al. (Glycoconjugate Journal 1, 37-42, 1984). The principal stages are briefly described below:

At each stage, the wells of a 96-well microplate are filled with 200 µl of the designated solution. The wells are first "coated" with a solution of GM2AP (prokaryotic recombinant protein) diluted to 50 ng/ml in a carbonate-bicarbonate buffer, pH 9.6. After incubating overnight at 4°C, the solution is removed and the wells are washed four times with PBS buffer pH 7.4 containing 0.05% Tween-20 (PBS-Tween). The microplates thus coated are stored at 4°C for about 2 weeks.

The urine samples at three different dilutions (20×, 40× and 80× or other appropriate dilutions) are incubated with an appropriate dilution of the anti-GM2AP or anti-Saposin B rabbit polyclonal antibody overnight at 4°C. A standard series of dilutions of a recombinant protein ranging from 2.0 to 62.5 ng/ml is used to prepare the calibration series and are treated in the same manner. All the dilutions are made in PBS-Tween buffer containing 1 mg/ml of ovalbumin. Thus, 0.2 ml of each incubated solution is added to "coated" wells in duplicate and the plates are left for 2 hours at room temperature. The wells are then washed four times in PBS-Tween and again filled with a solution of anti-rabbit IgG goat antibodies coupled to peroxidase and diluted about 1 200-fold. After incubating for 2 hours at room temperature, the wells are washed four times in PBS-Tween and again filled with the staining reagent. The staining reagent consists of 100 mg of 2,2'-azino-di-(3-ethylbenzothiazoline)sulfonic acid and 10 µl of 30% hydrogen peroxide for one hour at room temperature and the degree of staining of each microwell is estimated by reading the absorbance at 405 nm.

A standard curve is constructed by placing on the x-axis the concentration of GM2AP in the calibration series or of Saposin B with a logarithmic scale and on the y-axis the percentage absorbance with a linear

scale. The percentage absorbance of the sample is the absorbance ratio between the urine sample and the control which contains only the antiserum, without the soluble antigen.

A solution of recombinant protein GM2AP produced in a prokaryotic system, and having a concentration of 3 mg/ml, is diluted in 50 mM carbonate buffer, pH 9.6, and 50 µl are added to each well of a 96-well microplate, that is 50 µl per well of a solution at 0.5 µg/ml. The plates thus prepared are incubated overnight at room temperature. The anti-GM2AP polyclonal antibody produced in the laboratory (rabbit 79) was purified and diluted in PBS-0.05% Tween buffer in the presence of 10% horse serum. This solution is diluted 1/8 000. The solution is used to produce a calibration series with 8 series points covering concentrations from 0 to 500 ng/ml. A preincubation is carried out overnight at room temperature between 100 µl of antibody and 100 µl of urine sample to be assayed or of recombinant GM2AP or Saposin B protein solution serving for the calibration series. After washing the microplate in PBS-Tween, 50 µl of the incubation mixture are added per well, and then incubated for two hours at room temperature. The microplate is again washed in PBS-Tween, and then 50 µl of anti-rabbit IgG antibody coupled to peroxidase and diluted 1/5 000 are added to each microwell of the plate and incubated for two hours at room temperature. After further washings of the microplate, 100 µl of OPD are added to each well and incubated for 20 minutes at room temperature. The staining of each well, proportional to the concentration of GM2AP or of Saposin B recognized by the specific antibody used, is estimated by reading the absorbance.

A solution of recombinant protein GM2AP or Saposin B produced in a prokaryotic system, with a concentration of 3 mg/ml, is diluted in 50 mM carbonate buffer,

pH 9.6, and 50 µl are added to each well of a 96-well microplate, that is 50 µl per well of a solution at 1.5 µg/ml. The plates thus prepared are incubated overnight at room temperature. The purified anti-GM2AP peptides polyclonal antibodies produced in the laboratory (rabbit 190 and rabbit 191) are used alone or as a mixture, diluted 1/1 000 for each, in PBS-0.05% Tween buffer in the presence of 10% horse serum. The calibration series is produced using the prokaryotic recombinant protein GM2AP or Saposin B diluted so as to cover the concentration range 0 to 1 500 ng/ml with 8 points. 100 µl of antibody (one antibody or the two together) are preincubated in the presence of 100 µl of urine sample to be tested or of recombinant GM2AP or Saposin B solution, overnight at room temperature. After washing the microplate in PBS-Tween, 50 µl of the incubation mixture are added per well and then incubated for two hours at room temperature. The microplate is again washed in PBS-Tween, and then 50 µl of anti-rabbit IgG antibody coupled to peroxidase, diluted 1/5 000, are added to each microwell of the plate and incubated for two hours at room temperature. After washing the microplate, 100 µl of OPD are added to each well and incubated for 20 minutes at room temperature. The staining of each well, proportional to the concentration of GM2AP or Saposin B recognized by the specific antibody used, is estimated by reading the absorbance.

Example 15: Assay of the GM2AP proteins in the urines

The GM2AP protein was assayed in the urines of 22 patients suffering from multiple sclerosis (MS), 5 patients suffering from other neurological diseases (OND) and 9 individuals chosen from the active population and taken during a medical visit (healthy), according to the Elisa protocol described below, using anti-GM2AP polyclonal antibodies. The MS patients selected for this study are confirmed patients, that is

to say with various stages and profiles of the disease, and different treatments, and the like.

The results of the assay are presented in figure 8. Whereas only 0/5 OND urines and 2/9 so-called "Healthy" urines have a GM2AP concentration greater than 200 ng/ml, 10/22 (that is 45%) have a concentration greater than 200 ng/ml.

These results indicate that while the GM2AP protein is present in a very low concentration (<400 ng/ml) in the urines of individuals from the active population, it is present in higher concentration in the urines of MS patients. However, 12 MS urines also exhibit low levels of GM2AP. Among these 12 patients, 10 are under treatment. The high urinary concentrations of GM2AP appear to be a marker for the MS pathology, and more precisely a marker for one stage or one form of the disease, for the activity of the disease, and is certainly influenced by any ongoing treatment. It should be noted that two individuals in the active population have high GM2AP concentrations (these two cases were voluntarily included in the study, because they both exhibited a gliotoxic activity in their urines unlike the other individuals of this same category). It is impossible to know if they are healthy individuals, or individuals suffering from a pathological condition, or individuals suffering from multiple sclerosis because the samples from the so-called "Healthy" individuals were collected anonymously, with no knowledge of their clinical file.

Higher urinary concentrations of GM2AP are detected in the urines of MS patients; a high concentration of GM2AP can then be a marker for the MS pathology, and more precisely for one form of the disease, for one stage of the disease, or for a period of activity, and may be influenced by any ongoing treatment. These high urinary concentrations of GM2AP may also have a

predictive value for the onset of a worsening of the disease, or for a benign MS at the onset of a progression, and the like.

The absolute values of the GM2AP concentrations detected in the urines are dependent on the affinity and the specificity of the antibody used, but in general, the tendency between the three groups of individuals is preserved regardless of the antibody used.

Example 16: Assay of the Saposin B proteins in the urines

The Saposin B protein was detected in the same urine samples as those used to study the detection of GM2AP. The assays were carried out in parallel with those of GM2AP, in the same study, according to the Elisa protocol described below, using anti-Saposin B polyclonal antibodies.

The results of the Saposin B assay are presented in figure 9. 0/5 OND urines and 2/9 Healthy urines have a Saposin B concentration greater than 2 µg/ml, while 6/22 (that is 27%) exhibit a concentration greater than 2 µg/ml.

These results indicate that the Saposin B protein is present in each urine (so-called healthy population or so-called sick population) at significant concentrations, that is to say <2 µg/ml. These assay results are compatible with those described in the literature. However, even if Saposin B is present in each urine, it appears to be present in a higher concentration in certain MS urines. This increase in Saposin B concentration in the MS urines is perhaps masked by the basal concentration of this protein in the ordinary state. Thus, the high urinary concentrations of Saposin B appear to be a marker for the MS pathology,

and more precisely a marker for one stage or one form of the disease, or for the activity of the disease, and is certainly influenced by any ongoing treatment. The Saposin B assayed alone appears, however, to be a marker which discriminates for one form or for one activity of the disease slightly less than GM2AP. It should again be noted that two individuals from the active population have high Saposin B concentrations and they are the same individuals who also had a high GM2AP concentration in their urine.

In conclusion, higher urinary concentrations of Saposin B are detected in the urines of MS patients; a high Saposin B concentration can therefore be a marker for the MS pathology, and more precisely for one form of the disease, for one stage of the disease, or for a period of activity, and may be influenced by any ongoing treatment. These high urinary GM2AP concentrations may also have a predictive value for an onset of a worsening of the disease, or for a benign MS at the beginning of a progression, and the like. However, in general, the high Saposin B concentrations alone appear to be markers which are less discriminatory than high GM2AP concentrations alone.

The absolute values of the Saposin B concentrations detected in the urines are dependent on the affinity and specificity of the antibody used, but in general, the tendency between the three groups of individuals is preserved regardless of the antibody used.

Example 17: Coassay of the GM2AP and Saposin B proteins in the urines

Figure 10 presents the GM2AP concentrations assayed in the urine samples described in figure 5 relative to the Saposin B concentration assayed in these same samples and described in figure 6. The MS samples (dark

diamonds) and the OND and "Healthy" samples (white diamonds) are presented on this graph.

On this graph, it appears clearly that:

- the higher the GM2AP concentration in the urines, the higher the Saposin B concentration. (We have shown that it is not a general case with other proteins and that it does not indicate a renal disturbance, with the assay of creatinine in parallel for each of the samples tested);

- the high GM2AP and Saposin B concentrations are characteristic of the MS samples (with the exception of two urines from the active population, mentioned above). These joint high GM2AP and Saposin B concentrations are markers for the MS pathology, more precisely for a window of the disease (quadran on the right and at the top of the graph).

In conclusion, this analysis confirms that high urinary concentrations of GM2AP (>400 ng/ml) and of Saposin B (>2 µg/ml) are codetected in the urines of MS patients and may represent markers for the MS pathology, more precisely for one form of the disease, for one stage of the disease, or for a period of activity, and may be influenced by any ongoing treatment. It is advantageous to assay the two proteins in parallel in each sample, and to consider the two concentrations.

Assay of GM2AP and Saposin B in the urine of two patients in the form of kinetics

MS patient No. 1 - Progressive remittent form

Urines of this patient were collected during the progression of his disease. The patient was hospitalized on D0 for an attack. He was subjected on D1 to a flash of corticoids and was then monitored over

time from a clinical point of view (the flash provided clinical improvement). Figure 11 shows the profile for the assay of GM2AP and of Saposin B in these urines during the progression, and figure 12 shows the profile of the product of the two GM2AP and Saposin B concentrations, indicating a codetection of high concentrations. The high GM2AP and Saposin B concentrations at the time of the attack and hospitalization decrease gradually over time after the flash of corticoids up to 90 days.

MS Patient No. 2 - Progressive form

Urines of this patient were collected during the progression of his disease. The patient was hospitalized on D0 for an attack. He was subjected on D1 to a flash of Endoxan and was then monitored over time from a clinical point of view (the flash provided clinical improvement and at D60, signs of a worsening of the disease were observed). Figure 13 shows the profile for the assay of GM2AP and of Saposin B in these urines during the progression, and figure 14 shows the profile of the product of the two GM2AP and Saposin B concentrations, indicating a codetection of high concentrations. The high GM2AP and Saposin B concentrations at the time of the attack and hospitalization decrease gradually over time after the flash of Endoxan (also called cyclophosphamide) up to 23 days and appear to increase, becoming high at D60, thus showing a perfect correlation with the progression of the clinical signs.

These results confirm that:

- high concentrations of GM2AP and Saposin B in the urines are markers for the MS pathology, and in particular the codetection of high concentrations of the two proteins together (indicated by the product of the two concentrations);

- the high concentrations of GM2AP and Saposin B in the urines are markers for the activity of the disease (here during the attack) or are markers influenced by the immunosuppressive treatments such as corticoids and Endoxan which lower the concentrations.

This example illustrates the fact that these markers can be used, inter alia:

- to carry out a therapeutic monitoring of a patient and evaluate the therapeutic benefits of a treatment for a given patient; or

- to predict a worsening of the disease, predict an activity peak, and the like

- to decide on an anticipated therapeutic resumption based on the clinical signs

Example 18: Correlation between the detection of the MRP14, GM2AP and Saposin B proteins in the urines and the gliotoxicity measured in these urines

To verify a correlation between the presence of these proteins alone or in combination in the urines and the gliotoxicity of the urines, the concentrations of a protein of interest and the gliotoxicity of a sample of urines from patients suffering from multiple sclerosis (MS), from patients suffering from other neurological diseases (OND) and from individuals taken from the active population termed "Healthy" were assayed in parallel. Among the MS patients, patients are noted with various forms and stages of the disease, under treatment or otherwise, at various activities of the disease.

The MRP, GM2AP and Saposin B proteins were assayed in human urines according to the Elisa protocols described

above. The assays analyzed in this example are those described in the preceding examples. Each urine sample analyzed in Elisa was analyzed by the MTT test to measure the gliotoxicity of each sample. The gliotoxicity is expressed as a percentage of dead cells (estimated by colorimetry using tetrazolium salts) of a murine astrocyte cell line (CLTT1.1) after 48 hours of incubation in the presence of centrifuged urine.

Figure 15 represents the GM2AP concentration as a function of the gliotoxicity of the urines determined by the MTT test.

22 MS urines (gray diamonds), 5 OND urines (black diamonds) and 9 so-called "Healthy" urines (black diamonds) were presented on the graph. They are the same urines which were studied in examples 15 and 16. It is observed that all the control urines (OND and Healthy) have low levels of GM2AP (<400 ng/ml) and a low gliotoxicity (<15%), with the exception of a Healthy control urine (already commented upon in example 15) for which a high GM2AP concentration and gliotoxicity are observed.

The MS urines are divided into three subpopulations:

- urines with low GM2AP concentration (<400 ng/ml) and low gliotoxicity (<15%),

- urines with low GM2AP concentration (<400 ng/ml) and gliotoxicity (>15%), that is essentially 3 urines,

- urines at high GM2AP concentration (>400 ng/ml) and high gliotoxicity (>15%).

These three subpopulations perhaps indicate MS subpopulations, that is to say different forms or stages of the disease, different activities of the disease, different therapeutic benefits, and the like.

However, it can be noted that all the urines having a high GM2AP concentration also have a high gliotoxicity.

In conclusion, a correlation is observed between high urinary GM2AP concentration and gliotoxicity (all the urines with a high GM2AP concentration are gliotoxic (10/10), and all the urines with a low GM2AP concentration are not gliotoxic (<15%), with the exception of 3 urines/12 MS). This indicates the involvement of the GM2AP protein in the mechanism of gliotoxicity, alone or in combination, in its natural or modified form, but which is recognizable by an anti-GM2AP antibody. Furthermore, the codetection of a high GM2AP concentration in the urines and of a high gliotoxicity correlates with one subpopulation of patients suffering from MS.

Figure 16 represents the Saposin B concentration as a function of the gliotoxicity of the urines determined by the MTT test.

22 MS urines (gray diamonds), 5 OND urines (black diamonds) and 9 so-called "Healthy" urines (light gray diamonds) were presented on the graph. They are the same urines which were studied in examples 15 and 16. It is observed that the richer the urines are in Saposin B, the more gliotoxic they are. There is a fairly clear correlation between the Saposin B concentration and the gliotoxicity of the urines.

In conclusion: a correlation is observed between high urinary Saposin B concentration and gliotoxicity. This indicates involvement of the Saposin B protein in the mechanism of gliotoxicity, alone or in combination, in its natural or modified form, but which is recognizable by the anti-Saposin B antibody used for the assay.

Figure 17 represents the product of the GM2AP and Saposin B concentrations as a function of the gliotoxicity of the urines determined by the MTT test.

The 22 MS urines (gray diamonds), 5 OND urines (black diamonds) and 9 so-called "Healthy" urines (light gray diamonds) of examples 15 and 16 were presented in figure 17. The gliotoxicity of these urines is analyzed according to the product of the GM2AP and Saposin B concentrations, that is to say according to the codetection of the two proteins in the urines. A correlation is very clearly observed between the product of the two GM2AP and Saposin B concentrations and the gliotoxicity which is much higher than on considering only one protein. It is observed that 5/5 of the OND urines have a low product of GM2AP and Saposin B concentration and a low gliotoxicity; 8/9 "Healthy" urines have a low product of GM2AP and Saposin B concentration and/or a low gliotoxicity. On the other hand, essentially three subpopulations of MS urines are distinguished:

- urines at low GM2AP.Saposin B concentration and low gliotoxicity (<15%),

- urines at high GM2AP.Saposin B concentration and high gliotoxicity (>15%).

These two subpopulations perhaps indicate MS subpopulations, that is to say different forms or stages of the disease, different activities of the disease, different therapeutic benefits and the like. However, it is very important to note that all the urines having a high GM2AP and Saposin B concentration, that is to say having simultaneously a high GM2AP and Saposin B concentration, also have a high gliotoxicity. The two subpopulations of MS patients are all the more marked and clear when the three markers are considered together: gliotoxicity, high GM2AP concentration and

high Saposin B concentration. This is confirmed in figure 18.

In conclusion: a correlation is observed between high urinary GM2AP and Saposin B concentration and gliotoxicity. All the urines with a high GM2AP and Saposin B concentration are gliotoxic, and all the urines with a low GM2AP and Saposin B concentration are not gliotoxic (<15%), with the exception of 2 urines/22 MS. This indicates the involvement of the two proteins GM2AP and Saposin together or in combination in the mechanism of gliotoxicity, in their natural or modified form, but which is recognizable by the anti-GM2AP and anti-Saposin B antibodies used for the assay. Furthermore, the codetection of a high urinary GM2AP and Saposin B concentration and of a high gliotoxicity correlates with a subpopulation of patients suffering from MS (stage, form, activity, treatment of the disease?), compared with another subpopulation. These three markers considered together make it possible to discriminate between two subpopulations of MS patients.

Variation of the gliotoxicity and of the GM2AP and Saposin B concentrations as a function of the progression of the disease in two patients after and during treatment

The correlation between gliotoxicity, high GM2AP and Saposin concentration in the urines and MS pathology was also confirmed by measuring these three parameters in the urine of two patients during the progression of their disease.

Patient No. 1: MS remittent-progressive form, hospitalized on D0 for an attack and who had received a flash of corticoid on D1. After the flash, he showed a clinical improvement up to D90 - (cf. figures 11, 12),

Patient No. 2: MS progressive form, hospitalized on D0 for an attack and having received a flash of Endoxan (also called cyclophosphamide) on D1. On D60, he shows new clinical signs of a worsening of his disease - (cf. figures 13, 14).

The following were shown for the two patients:

- a correlation between the urinary gliotoxicity and the clinical progression of the disease (when the clinical signs are severe, the gliotoxicity is high; when the clinical signs decrease following the treatment, the gliotoxicity decreases and becomes stationary; when the signs of a worsening appear after the treatment, the gliotoxicity appears to increase again),

- a correlation between the gliotoxicity level in the urines of patients and the GM2AP and Saposin B concentrations, and

- a correlation between the high GM2AP and Saposin B concentrations and the clinical progression of the disease.

In conclusion: the assay of the GM2AP and Saposin B proteins in the urines is a good discriminatory marker for a subpopulation of MS (stage, form, activity, treatment of the disease). The GM2AP and/or Saposin B proteins are involved in the mechanism of gliotoxicity, alone or in combination, in their natural form or in a form which is recognizable by the polyclonal antibodies used for their assay. As the GM2AP and Saposin B proteins are codetected in high concentration in the gliotoxic urines, it is possible that these two proteins act in combination to induce the gliotoxicity.

Example 19: Immunohistochemical analysis of the expression of the GM2A, SAPB, MRP14 and MRP8 proteins

in a culture system producing gliotoxin in vitro (monocyte cultures), and in normal and pathological cerebral tissue for MS and for controls.

Protocol: Cultures of monocytes from a patient suffering from MS and from a healthy control were carried out in parallel, according to the present protocol described briefly. Starting with peripheral blood from these two volunteers collected over ACD, the PBMC (Peripheral Blood Mononuclear Cells) are isolated on Ficoll using the technique known to persons skilled in the art. The cells recovered (at the level of the ring) are washed twice in RPMI medium. The cells are then counted on Kovas slide and are inoculated in a primary bottle of 25 cm^2 or on Labteck slide (8 wells) (in permanox) in RPMI medium supplemented with 15% human AB serum on D0. The cells are cultured on "Labtek" type chamber slides in order to obtain a direct support for the analysis of the monocytes which adhere to the support and subsequently differentiate into macrophages. For the slides, 2×10^6 cells are then inoculated in an amount of 0.25×10^6 cells/well. For the bottles, 4×10^6 cells are inoculated in an amount of 0.25×10^6 cells/well. On D1, the cells in suspension are recovered and the Labteck wells or the bottles are washed twice with RPMI (previously heated to 37°C) before adding RPMI medium supplemented with 5% human AB serum. On D1, D3, D6, D9, D12 or 14, D15, the culture medium is changed; the supernatants are collected and the cells bound to the slides using the techniques known to persons skilled in the art. At each change of medium, at least two slides were fixed in paraformaldehyde and stored for the immunohistochemical analysis.

Composition of the medium: RPMI (500 ml) with 15 ml of 200 mM glutamate, 5 ml 100 µM sodium pyruvate, 5 ml of nonessential amino acids (100×), antibiotics penicillin

and streptomycin 100 000 U/µl and anti-human interferon antibodies at 100 U/µl.

Results: Four cultures of monocytes *in vitro* were thus studied in the form of kinetics: two cultures of monocytes derived from blood from control individuals and two cultures of monocytes derived from MS patients. At various culture times (D0, D1, D3, D6, D9, D12, was), the corresponding supernatants were also recovered. Once the kinetics was completed, the slides corresponding to the different days of culture were incubated in the presence of anti-GM2A, SAP-B, MRP-8 and MRP14 polyclonal antibodies. The gliotoxicity of each supernatant thus recovered was estimated by the MTT test. The concentration of GM2AP, MRP14 and Saposin B protein was also determined in each supernatant by the Elisa protocol as described in examples 13 and 14.

The immunofluorescence results on fixed cells are summarized below; it is possible to note:

- an absence of expression of MRP8 at all the stages of the 2 cultures

- a clear expression of MRP-14 in the period between D9 and D15, found in the two cultures, although higher in the MS culture. This expression appears to correlate with a macrophage differentiation stage.

- a very low expression (low intensity and low number of cells) is observed at the beginning of the culture in the control culture and probably corresponds to the physiological presence of GM2A in the macrophage lysosomes.

- In the MS culture, a much more marked expression of GM2A (greater intensity and larger number of cells) is observed, with a relatively homogeneous cytoplasmic

labeling between D3 and D6, disappears on D9 and is again noted on D14-D15 with an intense labeling localized at the cytoplasmic periphery, defining the inner contour of the plasma membrane. These observations are not found in all the control slides.

Analysis with the anti-SAP-B antibody did not make it possible to obtain an interpretable immunohistochemical labeling.

In the MS monocyte cultures already carried out, 3/3 had a gliotoxicity peak at D9 and 2/3 a smaller peak at D6, no peak being detected in the cultures of monocytes of 2/2 non-MS controls analyzed in parallel. Likewise, the assay of the MRP14, GM2AP and Saposin B proteins in the supernatant of the cell cultures during the kinetics showed that the SapB and GM2AP proteins are detected by Elisa in the supernatants of the MS monocytes and not in those of the control monocytes, on days D6 and especially D9 of the culture; the proteins are not detected beyond this kinetic. It should be noted that the antibodies used for the assay can recognize the physiological forms of the proteins, but also the complexed and/or modified forms.

It is therefore observed that the period D6-D9 during which the highest gliotoxicity is observed in the supernatant is covered by the period D3-D15 during which a less differentiated production of the negative control for GM2A is observed in the cells with quantitative and qualitative fluctuations of its cellular expression (quantity of expression and cellular localization).

Example 20: Immunohistological technique on brain sections in paraffin

The histological sections prepared in paraffin are made paraffin free in xylene and alcohol before undergoing a

pretreatment intended to unmask the antigens; this pretreatment may correspond to (i) twice 5 minutes under microwave (750W) in the presence of a sodium citrate, citric acid buffer, (ii) a treatment with acid by incubating for 15 minutes in a 1% periodic acid solution or by incubating for 5 minutes in a 99% formic acid solution. The endogenous peroxidases are then blocked by incubating the slides for 30 minutes in 1% hydrogen peroxide, followed by extensive washing in water for 15 minutes. The background noise is blocked by incubating the slides for 30 minutes in the presence of PBS-0.03% Triton, 10% Donkey serum (for the polyclonal antibodies) or 10% Goat serum (for the monoclonal antibodies). Labeling with the primary antibody is carried out by applying 100 to 200 µl of primary antibody solution per slide (0.5 to 5 µg/ml according to the titer) in PBS-0.03% Triton and then incubating for 2 hours at room temperature. The slides are then rinsed 3 times in PBS-Triton for 10 minutes. Secondary antibody labeling is carried out using biotinylated antibodies capable of binding specifically to the primary antibodies, for example anti-rabbit IgG or anti-mouse IgG antibodies diluted in PBS-0.03% Triton. The slides are washed and incubated in a solution for 2 hours (2 µl streptavidin-biotin-peroxide complex, 1 600 µl PBS-0.03% Triton). The slides are again washed before being revealed, protected from light, in buffer A and then rinsed with water before microscope observation. Buffer A for 5 slides: 25 ml 0.05M Tris, pH 7.6, 2.5 ml 1M Imidazole, 15 ml sterile water, 2 ml DAB 5 mg/ml, 5 ml 10% ammonium nickel, 30 µl 1% H_2O_2.

The same antibodies were used for an immuno-histochemical study, according to the technique briefly described below, on paraffined slides obtained by microtome section of brain collected post mortem from MS and from controls who had died from non-neurological pathologies.

The results of the analysis are summarized below:

There is no labeling of the "non-MS" and MS brains in the "normal" (non-lesioned) white substance and gray substance with the different anti-MRP8, MRP14 and GM2A antibodies. A nonspecific reactivity did not make it possible to interpret the results with the anti-saposin B antibody in this immunohistochemical application.

On the other hand, the following are noted in the plaque zones of MS brains:

- an anti-MRP14 reactivity in the macrophage and microglial cells, having a relatively homogeneous distribution over the entire stretch of the demyelination zones (plaques),

- a lower (less frequent) anti-MRP8 reactivity essentially linked to perivascular lymphoid infiltrates

- a clear anti-GM2A reactivity in the macrophages and microgliocytes of the plaque zones, with a particular density in the zones constituting the "glial wall" at the peripheral limit of a plaque. Labeling of a few astrocytes was also observed in the demyelination zones.

These different observations show that there is a particular hyperexpression of MRP-14 and GM2A proteins in the cultures of MS monocytes producing a gliotoxic activity in their supernatant, as well as in the zones defining demyelination plaques in the MS brains. They therefore reflect the reality of the coincidence between their abnormal coexpression, the production of gliotoxic activity and the demyelination lesions.

Furthermore, their abnormal production in the context of MS, in macrophage blood cells as well as in those of

the brain, indicates that it is justified to carry out their assay in biological fluids to correlate their quantity with the lesional and inflammatory activity of MS.

Example 21: Measurement of the activity of the T cells by proliferation of the T cells (Sredni et al., 1981).

The T cells are washed twice in culture medium in order to remove any trace of IL2 present in the initial culture medium. B lymphocytes (EBV-LCL) or monocytes/ macrophages taken as antigen-presenting cells are irradiated at 10 000 rads, and washed twice with culture medium (RPMI). 2×10^4 T cells (2×10^5 cells/ml) and 2×10^4 irradiated autologous B cells (2×10^5 cells/ml) are incubated together in the presence of an increasing antigen concentration range in a final volume of 200 µl in microwells. After 48 hours of culture at 37°C, 1 µCi of 3H-thymidine in 50 µl of RPMI medium is added to each well. The T cells, the only cells which divide, incorporate the tritiated thymidine into the DNA. After 18 hours of culture, the cells of each microwell are harvested on glass wool pastilles by aspiration. After osmotic lysis of the cells, the radioactivity incorporated into the DNA is absorbed onto the pastilles (cell Harvester 530, Inotech). Each dried pastille is placed in a plastic tube which contains 2 ml of scintillant; the radioactivity b adsorbed on each of the pastilles is quantified in a liquid scintillation beta counter (LKB Rackbeta 1217). The results are expressed as an arithmetic mean of cpm/culture ("counts per minute").

Example 22: Protocol for detecting the association between the peptides and the histocompatibility molecules (approach APC transformed with a peptide binding to MHC I).

1) Materials:

The sources of histocompatibility molecules are currently of two main types: mutant cells and purified histocompatibility molecules.

The mutant cell used is the human T2 cell which and a variant of the T1 line produced by fusion of the CEM T lymphoma and of the 721.174 B lymphoma (Salter and Cresswell Embo J 1986, 5: 943-949). This cell, which lacks peptide transporters, contains heavy chains of class I molecules free of peptides which will be able to accept exogenous peptides.

Class I histocompatibility molecules purified by affinity chromatography from human B cell lines transformed with EBV can also be used. In this case, the endogenous peptides should be removed by a treatment with 1.5 M urea and 12.5 mM sodium hydroxide (pH 11.7) for 1 hour at 4°C, followed by their removal by a desalting column (PDLO, Pharmacia). The histocompatibility molecules are immediately placed in contact with the peptides to be tested in a PBS buffer with 0.05% Tween 20, 2 mM EDTA, 0.1% NP40 and 6 mM CHAPS, in the presence of 2 µg/ml B2m to facilitate reassociation (Gnjatic et al., Eur J Immunol 1995 25: 1638-1642).

The peptides tested have in general 8 to 10 residues, sometimes 11 or 12. They were synthesized by Néosystems (Strasbourg), or by Chiron mimotopes (Victoria, Australia). They are used at concentrations varying from 100 µM to 0.1 nM.

2) Protocol for assembly (Connan et al., Eur J Immunol 1994, 24: 777; Couillin et al. Eur J Immunol 1995, 25: 728-732).

Aliquots of 8.105 cells in a volume of 64 µl, distributed in Eppendorf microfuge tubes, are brought into contact with a lysis buffer containing 10 mM PBS, pH 7.5, 1% NP40, protease inhibitors (1 mM PMSF, 100 µM iodoacetamide, 2 µg/ml aprotinin, 10 µM leupeptin, 10 µM pepstatin and 10 µg/ml trypsin inhibitor). The lysis is performed in the presence of the peptides to be tested for 30 minutes or 1 hour at 37°C. After removing the nonsolubilized material by centrifugation at 15 000 revolutions/minute at 4°C, the supernatant and supplemented with 140 µl of PBS containing 0.05% Tween 20, 3 mM of sodium azide, 1 mM PMSF and 10 mg/ml of bovine albumin. Each sample is incubated for 20 hours at 4°C in 2 wells of a microtiter plate of the Nunc type, Maxisorb, previously coated with a monoclonal antibody (10 µg/ml in PBS) which recognizes the histocompatibility molecules having conforming conformation(s) for the presentation of peptides and similar to that (those) present at the surface of the cells. The antibody-coated plate is saturated beforehand with bovine albumin at 10 mg/ml in PBS-Tween before placing the sample. The second antibody which allows the detection of the assembly of the histocompatibility molecules is directed against B2m. It is coupled either to biotin (NHS-LC biotin, Pierce) or to alkaline phosphatase (P-552, Sigma) and is incubated at 2 µg/ml for one hour at 37°C. In the case of the use of biotin, an incubation of 45 minutes at 20-25°C with streptavidin coupled to alkaline phosphatase (E-2636, Sigma) is carried out. The activity of alkaline phosphatase is measured using, as substrate, 4-methylumbelliferyl phosphate (M-8883, Sigma) at 100 µM in 50 mM diethanolamine, pH 9.5 with 1 mM $MgCl_2$. The reading is carried out at 340/460 nm with the aid of a cytofluorimeter.

3) Stability of the HLA/peptide complexes:

The stability of the abovementioned complexes was studied because it determines the good presentation of the antigen and the induction of the T response. To this effect, either purified HLA or the T2 cell lysate was used. With purified HLA, the endogenous peptides were removed (as described in 2)) and then it was brought into contact with the peptide to be tested in an Eppendorf tube at 37°C, for periods varying from a few minutes to several days. The following incubation phase on a 96-well plate (as described in 2) with the anti-HLA antibody is performed for one hour at 37°C. The revealing is carried out in a conventional manner. With the T2 cell lysate, all the incubations are also carried out at 37°C, after addition of all the protease inhibitors.